PACAP suppresses dry eye signs by stimulating tear secretion

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Dry eye syndrome is caused by a reduction in the volume or quality of tears. Here, we show that pituitary adenylate cyclase-activating polypeptide (PACAP)-null mice develop dry eye-like symptoms such as corneal keratinization and tear reduction. PACAP immunoreactivity is co-localized with a neuronal marker, and PACAP receptor (PAC1-R) immunoreactivity is observed in mouse infraorbital lacrimal gland acinar cells. PACAP eye drops stimulate tear secretion and increase cAMP and phosphorylated (p)-protein kinase A levels in the infraorbital lacrimal glands that could be inhibited by pre-treatment with a PAC1-R antagonist or an adenylate cyclase inhibitor. Moreover, these eye drops suppress corneal keratinization in PACAP-null mice. PACAP eye drops increase aquaporin 5 (AQP5) levels in the membrane and pAQP5 levels in the infraorbital lacrimal glands. AQP5 siRNA treatment of the infraorbital lacrimal gland attenuates PACAP-induced tear secretion. Based on these results, PACAP might be clinically useful to treat dry eye disorder.
Dry eye syndrome, also known as keratoconjunctivitis sicca, is a common eye disease caused by a reduction in the volume or quality of tears. Tear components are secreted from the main lacrimal gland, accessory lacrimal gland (Krause and Wolfring glands), meibomian gland, and the corneal and conjunctival epithelia in humans. A thin layer of tear film containing water, lipid electrolytes and ~10 mg ml\(^{-1}\) protein comprising different tear proteins, covers the ocular surface, thereby maintaining and protecting the eye. The major categories of dry eye are the aqueous tear-deficient type, in which the lacrimal glands fail to produce enough of the watery component of tears to maintain a tear film, and the evaporative type, in which impaired lipid secretion from the meibomian glands destabilizes the tear film\(^6\). Dry eye syndrome correlates with old age and affects females to a larger degree\(^2\). The number of patients diagnosed with the condition has increased in recent years, which could be due to the popularity of video display use (computer vision syndrome) or the wearing of contact lenses\(^3,4\).

The orthodox strategy for the treatment of dry eye syndrome is symptomatic therapy, such as tear replacement using artificial tears. Although artificial tears provide temporary symptomatic relief, they do not address the underlying pathophysiology of dry eye syndrome, and the outcome is not always satisfactory\(^5\).

Pituitary adenylate cyclase-activating polypeptide (PACAP; encoded by the gene Adcyap1), which exists in 27- or 38-amino-acid isoforms, was originally discovered in extracts of ovine hypothalamus\(^6,7\). The amino-acid sequence of PACAP—a member of the vasoactive intestinal polypeptide (VIP)/secretin/growth hormone-releasing hormone family of peptides—shows a 68% sequence homology with VIP. PACAP and VIP share three growth hormone-releasing hormone family of peptides—shows a unexpectedly discovered that some mice exhibited cloudiness of the cornea (Fig. 1a). The ocular surface appeared white and sandy, and blood vessels could be seen in the cornea (Fig. 1b). Based on fluorescein staining, which is commonly used to visualize corneal injury, strong fluorescence was observed in the central part of the cornea in these mice (Fig. 1c). On examination of this pathology, we discovered that the corneal epithelial cells were hypertrophied and the surface was keratinized (Fig. 1d). To quantify the degree of corneal keratinization, corneas were classified into four grades with the aid of a dissecting microscope (from Grade 0, denoting normal, to Grade 3 signifying hypertrophy of the surface and keratinization, as shown in Supplementary Fig. 1). Wild-type and Adcyap\(^+/−\) male mice over the age of 20 weeks had normal corneas, whereas about 40% of Adcyap\(^+/−\) male mice over 30 weeks of age had Grade 3 corneas (Fig. 1e). In female mice, all groups showed a higher frequency of keratinization than that observed in male mice (Fig. 1f). In female Adcyap\(^+/−\) mice, the percentage of animals showing corneal keratinization was <20% in animals younger than 10 weeks of age, but increased with age to 90% of animals over 30 weeks of age (Fig. 1f). These data indicate that corneal keratinization frequently occurs in older Adcyap\(^+/−\) mice, and particularly in female animals.

Because keratinization is a common feature of dry eye disorder, we postulated that the corneal keratinization was caused by a reduction in tear fluid or quality. To test this, the tear secretion level in Adcyap\(^+/−\) mice was measured using the cotton thread method. As expected, tear secretion levels of male and female Adcyap\(^+/−\) mice were reduced compared with those of wild-type mice aged 10 weeks or younger (Fig. 1g,h). The tear volume in eyes with corneal keratinization was significantly reduced compared with that of Grade 0 eyes (Fig. 1ii), while the tear volume and the corneal grade were weakly though significantly inversely correlated \((r = −0.242, P = 0.007,\) two-tailed Spearman’s correlation test). On histological examination, the infraorbital lacrimal gland, conjunctiva and corneal neural network of Adcyap\(^+/−\) mice were found to be morphologically normal (Supplementary Fig. 2a–c). Taken together, these observations suggest that Adcyap\(^+/−\) mice exhibit a dry eye-like phenotype with a reduction in tear volume and corneal damage, despite the structure of the infraorbital lacrimal gland, conjunctiva and neural network of the cornea remaining normal.

**Results**

**Dry eye-like signs in the Adcyap\(^+/−\) mouse.** During the routine housing of Adcyap\(^+/−\) mice in our animal facility, we unexpectedly discovered that some mice exhibited cloudiness of the cornea (Fig. 1a). The ocular surface appeared white and sandy, and blood vessels could be seen in the cornea (Fig. 1b). Based on fluorescein staining, which is commonly used to visualize corneal injury, strong fluorescence was observed in the central part of the cornea in these mice (Fig. 1c). On examination of this pathology, we discovered that the corneal epithelial cells were hypertrophied and the surface was keratinized (Fig. 1d). To quantify the degree of corneal keratinization, corneas were classified into four grades with the aid of a dissecting microscope (from Grade 0, denoting normal, to Grade 3 signifying hypertrophy of the surface and keratinization, as shown in Supplementary Fig. 1). Wild-type and Adcyap\(^+/−\) male mice over the age of 20 weeks had normal corneas, whereas about 40% of Adcyap\(^+/−\) male mice over 30 weeks of age had Grade 3 corneas (Fig. 1e). In female mice, all groups showed a higher frequency of keratinization than that observed in male mice (Fig. 1f). In female Adcyap\(^+/−\) mice, the percentage of animals showing corneal keratinization was <20% in animals younger than 10 weeks of age, but increased with age to 90% of animals over 30 weeks of age (Fig. 1f). These data indicate that corneal keratinization frequently occurs in older Adcyap\(^+/−\) mice, and particularly in female animals.

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**Distribution and function of PACAP in the lacrimal gland.** Based on the above data, we hypothesized that the PACAP/PAC1-R system was associated with altered tear secretion by the lacrimal gland, the major source of tear secretion. To test this, we first examined PACAP/PAC1-R expression and distribution in the mouse infraorbital lacrimal gland. Using the RT-PCR method, Adcyap1 and Adcyap1r1 mRNAs were detected in gland extracts, producing a signal with the same band size as that obtained from an eye ball sample that was used as a positive control (Fig. 2a). PACAP immunoreactivity was observed around acinar cells, and co-localized with immunoreactivity for the neuronal marker NeuN (Fig. 2b,c), and the parasympathetic neuronal marker choline acetyltransferase (ChAT) (Fig. 2d). The PACAP antibody recognized PACAP38 but not VIP (Supplementary Fig. 3a). PACAP38 was detected in wild-type, but not Adcyap\(^+/−\) mouse tears by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), and nanospray desorption electrospray ionization (nano-DESI) Orbitrap MS/MS (Supplementary Figs 4 and 5).
To investigate the function of PACAP in the lacrimal gland, PACAP38 was delivered in the form of eye drops to wild-type mice, and the level of tear secretion was measured using the cotton thread method (Fig. 3a). Eye drops containing $10^{-10}$ to $10^{-8}$ M PACAP38 significantly increased tear secretion from 15 to 45 min after treatment, with levels returning to baseline by 120 min (Fig. 3b). The basal tear secretion level and PACAP-induced tear secretion level did not differ significantly between males and females (Supplementary Fig. 6). PACAP27-containing eye drops also stimulated lacrimation, whereas the structurally related peptide VIP did not (Fig. 3c,d). Given that it has been reported that PACAP38, rather than PACAP27, is predominantly expressed in mammalian tissues, PACAP38 was used in the following experiments. When PACAP was administered unilaterally, tear secretion was only induced on the PACAP-treated side (Supplementary Fig. 7). When corneas were pre-treated with the topical anaesthetic Benoxil to suppress the corneal reflex, the basal lacrimation level decreased, but PACAP still elicited a significant increase in tear secretion (Supplementary Fig. 8). PACAP can thus induce tear secretion under topical anaesthesia, showing that it has a direct effect on the infraorbital lacrimal gland in the absence of the corneal/conjunctival reflex. Moreover, when the acute to semi-acute toxicological effect of PACAP38 (10^{-7} M) eye drops was evaluated at a concentration 1,000 times higher than the effective dose of PACAP38 (10^{-10} M) 48 h after the eye drop treatment, no morphological changes were observed in the corneas or in the infraorbital lacrimal glands (Supplementary Fig. 9). In addition, we examined the effect of PACAP on angiogenesis in vitro using human endothelial cells and fibroblasts in co-culture systems. No changes were observed in either the PACAP38 or PACAP6–38 (a PAC1-R and VPAC2-R antagonist)-treated groups (Supplementary Fig. 10). These data suggest that PACAP eye drops act locally to stimulate lacrimation without causing acute to semi-acute toxicity or eliciting a corneal reflex. Mice have two lacrimal glands (the infraorbital and exorbital glands) (Supplementary Fig. 11a), both of which were found to express PAC1-R and VPAC1-R (Supplementary...
Fig. 11b). Although PACAP still significantly stimulated lacrimation in a mouse model in which the exorbital lacrimal gland had been removed, it could not stimulate tear secretion in a second model in which both lacrimal glands had been removed (Fig. 3e,f). These findings indicate that the target organ for PACAP administered in an eye drop formulation is the infraorbital lacrimal gland.

We next examined the signalling cascade associated with PACAP-induced lacrimation. Pre-treatment with PACAP6–38 significantly suppressed PACAP-induced tear secretion (Fig. 3g), whereas VIP6–28 (a VPAC1-R and VPAC2-R antagonist) did not suppress tear secretion (Fig. 3h). Moreover, a single drop of PACAP6–38 (10⁻⁸ M) to the ocular surface reduced the level of normal lacrimation at the 15 and 60 min time points post-administration (Fig. 3g). The intravenous infusion of PACAP also increased tear secretion in a manner that could be inhibited by co-treatment with PACAP6–38 (Supplementary Fig. 12). These results indicate that PACAP eye drops stimulate lacrimation via an action on PAC1-R.

PACAP eye drops to Adcyap1⁻/⁻ mice. We also used PACAP-containing eye drops on Adcyap1⁻/⁻ mice. PACAP38 (10⁻¹⁶ M) dropped increased tear secretion in these mice as well as in their wild-type counterparts, suggesting that PACAP transiently restores tear secretion in Adcyap1⁻/⁻ mice (Fig. 4a). We subsequently tested the effects of repeated administration of PACAP38 on the eyes of Adcyap1⁻/⁻ mice (one eye treated with PACAP38, the other with saline) with a view to preventing corneal keratinization. After 3 weeks of treatment, the injury score had increased in saline-treated eyes, but was still at the pre-treatment level in PACAP-treated eyes (Fig. 4b,c). Angiogenesis and ocular hyperaemia were not observed in PACAP-treated eyes (Fig. 4b).

Signalling associated with PACAP-induced tear secretion. To determine the pathway related to PACAP-induced tear secretion, we next investigated the signalling pathways downstream of PAC1-R, focusing on the adenylate cyclase (AC)-cAMP-dependent pathway. As determined by ELISA, the CAMP level in mouse infraorbital lacrimal glands was increased at 15 min and peaked 30 min after the application of PACAP38-containing eye drops (Fig. 5a). The signal for phosphorylated (p) protein kinase A (PKA), a cAMP-dependent protein kinase, was significantly increased at 30 min (Fig. 5b), while pre-treatment with the AC inhibitor SQ22536 or with PACAP6–38 7.5 min before the administration of PACAP eye drops significantly suppressed the PACAP-induced phosphorylation of PKA (Fig. 5c). Pretreatment with the AC inhibitor dramatically suppressed PACAP-induced tear secretion (Fig. 5d).

Aquaporin expression in wild-type and Adcyap1⁻/⁻ mice. The aquaporins (AQP)s are a family of water channel proteins that are expressed in numerous tissues and organs, with expression of the AQP4 and AQP5 subtypes being reported in the lacrimal gland18. To evaluate the relationship between PACAP-induced tear secretion and AQP5, we examined AQP4 and AQP5 immunoreactivities in the infraorbital lacrimal gland in wild-type and Adcyap1⁻/⁻ mice. AQP5 immunoreactivity was identified on the apical side of acinar cells in wild-type mice, but only weak immunoreactivity was observed in these cells in Adcyap1⁻/⁻ mice (Fig. 6a). AQP4 immunoreactivity was observed on the basal side of acinar cells in both wild-type and Adcyap1⁻/⁻ mice, without any obvious difference between the two (Fig. 6a). The specificity of the AQP4 and AQP5 immunoreactivities was confirmed using an antigen pre-absorption test for AQP5 antibody or comparison with a primary antibody-free (AQP4 antibody) negative control (Supplementary Fig. 3d,e). On immunoblotting, the AQP5 signal was found to be significantly lower in the infraorbital lacrimal glands of Adcyap1⁻/⁻ mice than in those of wild-type animals, but the AQP4 signal was almost the same in both cases (Fig. 6b).

The trafficking of AQP5 protein from the cytosol to the membrane contributes to increased water permeability9. After fractionation and immunoblotting, the AQP5 signal in the membrane fraction was significantly lower in Adcyap1⁻/⁻ mice than in wild-type mice, but was not significantly different in the cytosolic fraction (Fig. 6c). In contrast, the AQP4 signals in the cytosolic and membrane fractions were similar for the two groups (Fig. 6c).

AQP5 expression and distribution after PACAP eye drops. The phosphorylation of AQP5 has been postulated to initiate its trafficking to the membrane20,21. On this basis, the cellular localization and degree of phosphorylation of AQP5 was evaluated in PACAP-treated infraorbital lacrimal glands. Thirty minutes after treatment with 10⁻¹⁰ M PACAP, AQP5 immunoreactivity on the apical side of acinar cells was greater...
than that in the saline-treated, SQ22536-pre-treated or PACAP6–38-pre-treated groups (Fig. 7a,b). The AQP5 levels in the total lysates of the infraorbital lacrimal gland extracts showed no difference between the groups at 30 min (Fig. 7c). However, the AQP5 signal in intraorbital lacrimal gland extracts immunoprecipitated with a pan-phospho antibody was clearly detectable in the PACAP-treated group, but was less obvious in the other groups (Fig. 7c). An AQP4 signal was not detected in the sample immunoprecipitated with the pan-phospho antibody (Supplementary Fig. 13). The AQP5 signals in the membrane fractions were increased 30 min after treatment with PACAP compared with the other groups (Fig. 7d,e). To elucidate the contribution of AQP5 to PACAP-induced tear secretion, an AQP5 gene-silencing experiment was...
When infraorbital lacrimal glands were treated with AQP5 siRNAs, the Aqp5 mRNA level was significantly decreased by about 70%, whereas the Aqp4 mRNA level remained almost the same 24 h after the siRNA treatment compared with the control group (Fig. 8b,c). AQP5 siRNA treatment reduced AQP5 immunoreactivity in the infraorbital lacrimal glands, but not AQP4 immunoreactivity (Fig. 8d). The AQP5 siRNA treatment significantly decreased the basal level of tear secretion, and PACAP-induced tear secretion was attenuated 24 h after the siRNA treatment (Fig. 8e).

Taken together, these findings suggest an underlying mechanism whereby PACAP and its receptor are expressed in mouse infraorbital lacrimal glands. PACAP stimulates tear secretion via an AC/cAMP/PKA cascade, which in turn stimulates AQP5 translocation from the cytosol to the membrane of lacrimal acinar cells to bring about an increase in water permeability (Fig. 8f).

Discussion
Dry eye syndrome is more common in women than in men, particularly in older patients. This study has made use of the

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**Figure 4** | Effect of PACAP eye drops on female Adcyap1−/− mice. (a) Tear secretion level in Adcyap1−/− mice after application of saline or 10−10 M PACAP38 eye drops (n = 9 per group). *P < 0.05 versus saline-treated group. (b,c) Corneal scoring before and after the repeated application of PACAP eye drops to Adcyap1−/− mice over a 3-week period. (b) Representative images of the corneal surface before and after eye drop application. Scale bar, 2 mm. (c) Eighteen female Adcyap1−/− mice were given saline or PACAP eye drops unilaterally. The classification of each side of the cornea was evaluated separately before, and 3 weeks after eye drop treatment commenced. *P < 0.05.

**Figure 5** | Contribution of the cAMP/PKA pathway to PACAP-induced tear secretion. (a) cAMP content in infraorbital lacrimal gland extracts after application of 10−10 M PACAP eye drops (n = 8 per group). *P < 0.05 versus 0 min. (b) Phosphorylated PKA (pPKA) levels in the mouse infraorbital lacrimal gland after PACAP eye drop application (n = 6 per group). *P < 0.05 versus 0 min. (c) Pre-treatment with the adenylate cyclase inhibitor SQ22536 or the PACAP receptor antagonist PACAP6−38 suppressed PKA phosphorylation 30 min after PACAP eye drop administration (n = 6 per group). *P < 0.05. (d) Effect of SQ22536 pre-treatment on PACAP-induced tear secretion in male mice (n = 10 per group). A single drop of test solution was applied to each ocular surface. The sum of the wet lengths of thread from both eyes is shown. *P < 0.05, **P < 0.01 versus saline + PACAP group.
Figure 6 | AQP4 and AQP5 levels in the infraorbital lacrimal glands of male wild-type and Adcyap1−/− mice. (a) AQP4 and AQP5 immunoreactivities in wild-type and Adcyap1−/− mouse infraorbital lacrimal glands. Scale bar, 50 μm. (b) AQP4 and AQP5 signals in infraorbital lacrimal gland total lysates from wild-type and Adcyap1−/− mice detected by western blot analysis and expressed as semi-quantified results ($n = 6$ per group). *$P<0.05$ versus wild-type mice. (c) AQP4 and AQP5 signals from fractionated infraorbital lacrimal glands and the semi-quantified results for wild-type and Adcyap1−/− mice ($n = 6$ per group). *$P<0.05$ versus wild type. GAPDH and pan-cadherin signals were used as internal controls for cytosolic and membrane protein, respectively.

Figure 7 | PACAP eye drop-induced phosphorylation and trafficking of AQP5. (a) Representative images of AQP5 immunoreactivities in infraorbital lacrimal gland acinar cells after the application of saline, PACAP38, PACAP38 + SQ22536 (SQ) or PACAP38 + PACAP6–38 eye drops. Scale bar, 10 μm. (b) AQP5 signals in fractionated infraorbital lacrimal glands and the semi-quantified results ($n = 11$ per group). **$P<0.01$. (c) AQP5 signals in total lysate and immunoprecipitation with a pan-phospho antibody. Immunoprecipitation with a normal IgG antibody was used as a negative control. (d,e) AQP5 signals in fractionated infraorbital lacrimal glands (d) and semi-quantified results (e) ($n = 11$ animals per group). *$P<0.05$
discovery of a new Adcyap1$^{-/-}$ mouse phenotype, namely the manifestation of corneal keratinization, which was particularly apparent in female mice as a function of age, the overall implication being that Adcyap1$^{-/-}$ mice manifest dry eye-like signs. The tear-secreting response to PACAP eye drops and the basal level of tear secretion were, nevertheless, similar between male and female wild-type mice (Supplementary Fig. 6). It has been suggested that the sex difference in dry eye syndrome in male and female wild-type mice (Supplementary Fig. 6). It has been reported that the sex difference in dry eye syndrome in human is due to the influence of female sex hormone levels. It has been suggested that the sex difference in dry eye syndrome in humans is due to the influence of female sex hormone levels. Given that female Adcyap1$^{-/-}$ mice exhibit decreased serum progesterone levels\(^2\), dry eye-like symptoms in these animals may be due to an imbalance in their sex hormone levels.

Animal models that mimic dry eye disorder have been established by several groups\(^2\). One type of dry eye is the aqueous-deficient model, which mimics the dry eye symptoms caused by autoimmune diseases such as Sjögren’s syndrome, removal or irradiation of the lacrimal gland, or neuronal pathway dysfunction. Another type is the evaporative dry eye model, as such as exposure to a dry environment, and pharmaceutically induced tear film instability caused by a decrease in oil or mucin secretion in the tears as evidenced by dry eye symptoms. However, a dry eye model arising from a specific endocrine imbalance has not been reported to date. We have shown that Adcyap1$^{-/-}$ mice exhibit (1) reduced lacrimation when left untreated, (2) increased lacrimation upon PACAP administration and (3) a morphologically normal lacrimal gland.

These findings suggest that the impairment of lacrimal secretion in Adcyap1$^{-/-}$ mice results from lacrimal gland dysfunction rather than developmental or structural abnormalities. Moreover, Adcyap1$^{-/-}$ mice spontaneously develop corneal keratinization with aging, implying that this mouse phenotype could serve as a novel non-Sjögren’s type aqueous-deficient dry eye model arising from lacrimal gland dysfunction.

AQP family genes and proteins are expressed in the eye and its accessory organs. It has been reported that AQP5 immunoreactivity is markedly decreased in the lacrimal acinar cells of people with Sjögren’s syndrome, a chronic autoimmune disorder with impairment of the moisture-producing glands. The reduced expression of AQP5 suggests that this protein may be related to the decrease in tear secretion in this disease. On the other hand, Verkman’s group reported that AQP5 KO mice do not exhibit an altered tear volume. However, the same group recently published data showing that the Na$^+$ content of tears from AQP5 KO mice is significantly higher than that of wild-type mice, suggesting that the ion concentration in AQP5 KO mouse tears is elevated due to decreased water secretion into the tear fluid. In our study, AQP5 gene silencing attenuated PACAP-induced tear secretion, as well as the basal level of tear secretion. Taken together, we propose that AQP5 is associated with water secretion into the tear.

It has been reported that the activation of cAMP/PKA can induce the translocation of AQP5 from the cytosol to the apical membrane. Moreover, X-ray analysis of the structure of human AQP5 has revealed that phosphorylation at the C-terminal is required for the conformational change for trafficking. Although the relationship between membrane trafficking and phosphorylation of AQP5 is not yet fully understood, in the case of AQP2, the closest paralog of AQP5,
a key event for membrane trafficking is the phosphorylation of a C-terminal site by PKA (refs 33,34). Here, we demonstrate that PACAP eye drops induce an elevation of cAMP, pPKA and pAQP5 levels and membrane trafficking of AQP5 in the mouse infraorbital lacrimal gland, suggesting that PACAP is a regulator of AQP5 trafficking in this gland.

Gilbard and collaborators reported that topical administration of a cAMP inducer such as VIP stimulates tear secretion in a rabbit model of keratoconjunctivitis sicca35 and in patients with dry eye disease36. However, they did not further study the nature of the target organ, or how reagents stimulate tear secretion. Here, we have demonstrated that PACAP stimulates tear secretion from the infraorbital lacrimal gland via a PAC1-R/cAMP/PKA/AQP5 cascade. Moreover, PACAP had a much greater effect on tear secretion than VIP. It should be noted that the Gilbard study used a high concentration of VIP (2 × 10⁻⁶ M) as eye drops in their rabbit model, whereas we used 10⁻⁷ M and 10⁻⁸ M, the same as our effective doses of PACAP in mice. We also found that PAC1-R is the main receptor for PACAP-induced tear secretion. The affinity of PACAP for PAC1-R is 1,000 times higher than that of VIP, implying that the higher concentration of VIP may be able to stimulate tear secretion via PAC1-R. Taken together, these results suggest that cAMP signalling is an important step for tear secretion, and that PACAP is an endogenous tear regulator in the lacrimal gland, with much greater potential than VIP to stimulate lacrimation.

Tear fluid includes several antibacterial proteins, growth factors and secretory mucin for corneal maintenance37,38. As it has been reported that systemic infusion of PACAP alters the composition of tears, especially the keratin family of proteins in rats39, PACAP may regulate tear protein secretion as well as tear fluid secretion. It is also well known that tear secretion is important for corneal healing40, and for this reason we postulated that a reduction in tear fluid would be an important factor underlying corneal keratinization in the Adcyap1⁻/⁻ mice, and that PACAP could protect the corneal surface by stimulating tear secretion. Moreover, we used MALDI-TOF MS and nano-DESI MS/MS to identify the presence of PACAP in mouse tear fluid, a finding that may imply that PACAP is secreted from the lacrimal gland into the tear fluid, thereby directly affecting the cornea. However, it remains questionable from which tissues in tear fluid PACAP is derived. The PACAP could be coming from corneal and conjunctival nerve endings released by dromic (parasympathetic or sympathetic nerves) or anti-dromic (sensory nerves) stimulation, conjunctival goblet cells or stratified squamous cells, or the meibomian glands. If the corneal keratinization in Adcyap1⁻/⁻ mice is due to evaporative water loss caused by a reduced volume of tears, it could be postulated that lid closure (for example, reversible closure, using cyanoacrylate glue) in Adcyap1⁻/⁻ mice would inhibit the development of this phenotype. Future studies will be needed to clarify the source of PACAP in tear fluid and the corneal healing effects of PACAP.

Although our findings indicate the potential of PACAP as a stimulator of tear production in mice, there are still problems that would need to be resolved in relation to drug development. First, the human and mouse lacrimal apparatuses are structurally different. In mice, the exobital lacrimal glands are located near the tear excretory ducts into the eyelid. The intraorbital lacrimal glands and the lipid-secreting Harderian glands are located in the orbit of the eye. In humans, one main lacrimal gland is located in the lower eyelid fossa of each orbit next to the eye ball; this connects with excretory ducts in the upper fornix. However, over 50 accessory lacrimal glands are scattered over the inner surfaces of the lower and upper eyelids, and the lipid-secreting meibomian gland is located in the tarsal plate of the upper and lower eyelids41. We anticipate that PACAP eye drops will not reach the main lacrimal gland in humans because of its location, making the target of PACAP the accessory lacrimal glands. However, it is still an open question whether PACAP only affects the lacrimal gland, or whether the corneal and conjunctival epithelia, and the intraorbital lipid-secreting gland are also involved. In any future clinical trials of PACAP on dry eye patients, it will be important to clarify the expression of PACAP and PACAP receptors in tear-secreting tissue, including the lacrimal glands, as well as the effect and target of PACAP eye drops in humans. Moreover, we showed that PACAP eye drops did not cause any adverse reaction in acute to semi-acute phase at a concentration of 10⁻⁷ M (Supplementary Fig. 9), or in chronic eye drop treatment at a concentration of 10⁻¹⁰ M for 3 weeks in Adcyap1⁻/⁻ mice (Fig. 4). However, these toxicological evaluations may not be sufficient for safety trials of PACAP eye drops to proceed on healthy volunteers. Further suitable toxicological tests will be required before a clinical trial.

In this study, PACAP38 eye drops at the lower dose of 10⁻¹⁰ M stimulated mouse lacrimation for <1 h. Even if the effects of PACAP are similar in mice and humans, this short period of action would pose a problem in a clinical setting. We believe that the short-acting effect of PACAP in our study was due to the PACAP eye drops being washed from the ocular surface by lacrimation. One solution could be to formulate an ointment to provide sustained release. However, low doses of bioactive peptides have both advantages and disadvantages in terms of drug development. A low dose of peptide-based medicine offers good efficacy, safety and high selectivity and potency. In contrast, there are issues in relation to instability, short half-life and rapid elimination42. These problems would need to be overcome if PACAP were to be developed as an effective stimulator of lacrimation.

In conclusion, our results highlight a new function of PACAP as a stimulator of tear production initiated via the PAC1-R/AC/cAMP/PKA/AQP5 cascade. We found that PACAP eye drops induce tear secretion and suppress the progression of corneal keratinization in Adcyap1⁻/⁻ mice. Topical administration of cyclosporine has been developed for dry eye patients to provide an anti-inflammatory effect; however, eye drops focusing on tear-stimulating mechanisms are still in the developmental stage. The findings from our work are encouraging and should provide the impetus for further preclinical and clinical studies on the efficacy of PACAP eye drops to treat dry eye patients.

Methods

Animals. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University (08116, 09110, 50017, 51033, 510043, 52007, 52013, 53020, 53025). The Adcyap1⁻/⁻ mice on the C57Bl/6 background were established by Dr Akemichi Baba43 and were bred and maintained under specific pathogen-free conditions in the animal facility of Showa University. Animals were housed in a facility with a 12-h/12-h light/dark cycle and were given free access to water and standard rodent chow. Eight to twelve weeks old mice were used, excepting Figs 1 and 4, and Supplementary Figs 1 and 2 with 8 to over 30 weeks old mice. In this study, both male and female mice were used as described in the figure legends.

Evaluation of corneal scoring. Keratinization of the cornea was classified into four grades (Grades 0–3) by visual observation under a dissecting microscope as follows: Grade 0 (normal) = no observable abnormality; Grade 1 = clouded cornea; Grade 2 = angiogenesis; and Grade 3 = hypertrophy of the corneal epithelium as shown in Supplementary Fig. 1a–d. The evaluation of corneal score was performed in a blinded fashion. The corneal keratinization of wild-type, Adcyap1⁻/+ mice, and Adcyap1⁻/⁻ mice was evaluated on both sides, and the higher grade was used for the grading of the animals in Fig. 1e.f.

Corneal fluorescein staining. Corneal injuries were visualized with fluorescein staining. Adcyap1⁻/⁻ and wild-type mice were anaesthetized with an
intrapеритонеальное (i.p.) инъекции пентобарбитала, и 2 мл флуороценина solution (1 μM) растворённых в воде, было нанесено на глаза через катаракту. После прекращения животных инъекции, остатки раствора были удалены с помощью гигиенического средства. Фотографии были получены с цифровой камерой (CAMEO CS050, Olympus, Tokyo, Japan) и предоставлены для анализа стереоскопического микроскопа.

**PACAP-containing eye drops and cotton thread tear test.** The rate of lacrimal secretion was determined with the cotton thread test using standardized phenol red-impregnated cotton threads (Zoe-Quek; Menicon Co. Ltd., Nagoya, Japan). Mice were anaesthetized with an i.p. injection of pentobarbital to prevent autonomic nervous system reflexes that could have affected tear secretion. The baseline rate of tear secretion was evaluated by insertion of a thread through the lower eyelid for 30 s, after which the blink frequency of the changed thread that had absorbed the tear fluid was measured in millimeters. The test solution, PACAP38 (10−6–10−12 M, Peptide Institute, Osaka, Japan), PACAP27 (10−6−10−8 M, Peptide Institute), VIP (10−8 M or 10−10 M, Peptide Institute) or saline was then applied to both ocular surfaces. The thread was then removed to permit blinking five times, and the thread was then reinserted. Repeated application of the drop was performed after 7.5 min, and the experiment was continued for the subsequent tear secretions to prevent drying of the surface of the cornea.

**Histology.** Adult C57BL/6J mice (Charles River Japan, Kanagawa, Japan) were anaesthetized with sodium pentobarbital (50 mg kg−1, i.p.) and perfused with phosphate-buffered saline (PBS) followed by 2% paraformaldehyde in PBS. Immediately afterwards, the infraorbital lacrimal glands were removed and fixed in the same fixative for 24 h at 4°C. Fixed tissues were embedded in paraffin for histopathological studies with hematoxylin and eosin staining, or frozen sections were used for double-labeling immunofluorescence staining.

**Toxicology of the PACAP eye drops.** In acute to semi-acute toxicology experiments, 2 μl of 10−5 M PACAP or saline were applied as eye drops, and the eye was used for the adnexal tissues of a treated animal to avoid false positive 48 h later. The acute to semi-acute toxicological effects of PACAP on the cornea and infraorbital lacrimal gland were evaluated based on hematoxylin and eosin staining.

**Double-labeling immunofluorescence staining.** The following primary antibodies were used according to standard protocols41. Eight-jm-thick frozen sections were obtained in 5% normal horse serum for 1 h and incubated overnight at 4°C in phosphate-buffered saline (PBS) with 0.1 M NaCl and 1% Triton X-100) with a protease inhibitor cocktail (Sigma). Immediately afterwards, the infraorbital lacrimal gland sections were fixed and stained in the same Fixative for 24 h at 4°C. Fixed tissues were embedded in paraffin for histopathological studies with hematoxylin and eosin staining, or frozen sections were used for double-labeling immunofluorescence staining.

**Repeatead eye drop study.** Adcyop1+/− female mice were given eye drops containing 10−10 M PACAP or saline, 2 μl per eye (unilaterally), 2 times per day, 6 days per week for 3 weeks. We selected 18 female Adcyop1+/− mice based on similar average pre-treatment corneal keratinization scores on both sides. The scoring method was the same as the previous evaluation for each side separately.

**cAMP enzyme immunoassay.** Enzyme-linked immunoassays for cAMP were performed using a cAMP enzyme immunoassay (EIA) kit (Camyan Chemicals, Grand Rapids, MI, USA) following the manufacturer’s instructions. In brief, C57BL6J mice were anaesthetized, and 2 μl of 10−6 M PACAP was applied to both ocular surfaces. Both infraorbital lacrimal glands were removed at 7.5, 15 or 30 min after the application of the eye drops (n = 8). They were then homogenized in 200 μl of 5% trifluorooracetic acid on ice. After centrifugation at 1,500g for 10 min, the lysate was mixed with 1 μl of ether for 10 s. After removal of the ether, the aqueous layer was acetylated according to the ELISA kit manufacturer’s instructions and used for the EIA assay. The absorption in each well was measured with a plate reader (POLARstar Omega; BMG LABTECH GmbH, Offenburg, Germany).

**Immunoblotting.** For western blot analysis, mice were euthanized by decapitation and their infraorbital lacrimal glands were immediately removed. The infraorbital lacrimal glands were then homogenized in cold lysis buffer (10 mM Tris-HCl, 0.15 M NaCl and 1% Triton X-100) with a protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 12,000g for 30 min at 4°C and the resulting supernatant was the total cell lysate, which was subsequently diluted with SDS sample buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 40% glycerol, 4% 2-mercaptoethanol and 0.002% bromophenol blue) and incubated for 12 h at 4°C. This lysate sample (30 μg) was electrophoresed on a 7.5% polyacrylamide gel containing 0.1% SDS at 100 V. The protein bands were then transferred from the gel to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 3 h at 100 mA. The membrane was initially blocked with 2% Blockace (Dainihon Pharmaceuticals, Osaka, Japan) at Tris-buffered saline with Tween 20 (TBS-T) for 1 h at room temperature and probed overnight with a mouse monoclonal antibody for β-actin (product size 450 bp) mixed with the EX taq kit (Takara Bio, Otsu, Japan) with the PCR conditions of preheating for 15 s at 95°C, annealing at 50°C for 30 s, and elongation at 72°C for 1 min with a final extension at 72°C for 10 min. The PCR products were separated by gel electrophoresis on an agarose gels and visualized with an illuminator. The gel images were cropped for presentation. The uncropped images in Figs 2a–7 are shown in Supplementary Fig. 14.

**Real-time PCR.** RNA isolation was followed by the reverse transcriptase PCR method. Reverse transcription into complementary DNA was achieved using the reagents and protocol of the PrimeScript RT reagent kit (TaKaRa BIO, Kyoto, Japan). The PCR primer set was as follows: mouse Adcyop4 primers, forward: 5′-GAG GTC GCT TCG CAC CAG-3′, reverse: 5′-TTG ATG GTG TGT TCC CAG CAC-3′ (product size 487 bp), mouse Vpa2r1 primers, forward: 5′-GCA AGC GCA GCC GTT GCT GCT CTG-3′ (product size 498 bp), and mouse Actb primers: forward: 5′-GGC AAG GTC ATC CAT GAC AAC-3′, reverse: 5′-GTC CAC CAC CCT GTT GCT GTA-3′ (product size 498 bp). Relative gene expression was calculated using the comparative Ct method with the Ct values of the housekeeping gene, Gapdh. mRNA levels were normalized, with the percentage for control groups taken as 100%.
**Immunoprecipitation.** All immunoprecipitations were carried out using the immunoprecipitation kit Catch and Release v2.0 (Millipore) following the manufacturer’s instructions. In brief, mouse infraorbital lacrimal gland lysate was obtained 30 min after the application of eye drops. Four-hundred microgram protein from the cell lysate, 1 µg of mouse anti-ß-Thy, ß-Thy, or ß-Thr (pan-phospho) IgG antibody (AnaSpec Inc, San Jose, CA, USA) for the capture antibody or a normal mouse IgG (Millipore) as the control antibody, and 10 µl of the antibody capture affinity ligand (total 500 µl) were mixed and incubated in Catch and Release v2.0 spin column containing prepacked immunoprecipitation capture resin. After end-over-end shaking for 16 h at 4°C, the column was centrifuged, washed 3 times and then eluted with 70 µl of the elution buffer. The eluent was analysed by immunoblotting.

**Removal of lacrimal glands.** Under inhalation anaesthesia with sevoflurane, the hair from under the eye to the outer canthus of the eyes was shaved, and a 15 mm incision was made in the infraorbital lacrimal gland removal model, the exorbital lacrimal gland, which is located under the ear, was exposed and removed. In the infraorbital and exorbital lacrimal gland removal model, the exorbital lacrimal gland and the lacrimal duct were first isolated. The infraorbital lacrimal gland was exposed by pulling the lacrimal duct, after which both lacrimal glands were removed. The skin was then sutured and the animals were kept warm during their recovery from anaesthesia. The tear secretion level was checked before and after surgery.

**Semi-quantification of AQP5 immunoreactivity in lacrimal acini.** The densities of AQP5 immunoreactivity in the mouse infraorbital lacrimal gland 30 min after administration of eye drops containing saline, PACAP38, PACAP38 + SQ22536, or PACAP38 + PACAP6–38 was evaluated. AQP5 immunostaining was performed following the above method. One hundred pictures of acini from 10 infraorbital lacrimal glands (10 acinus pictures/lacrimal gland) from five wild-type mice in each group were cut out with grey scale. Using Image J software (ver. 1.44p), the average density in the apical membrane, and in four spots of 1 µm² in the cytosol, area were measured. The value of the apical membrane density was determined using the cytoksolic density value as background. The quantification was performed with a blinded test, masking sample data for another person who used Image J.

**AQP5 siRNA treatment in vivo.** An HPLC grade of non-target negative control and three types of mouse AQP5 siRNAs (sequence was shown in Supplementary Table 1) were designed and purchased from BONAC Corporation (Fukuoka, Japan). Mouse AQP5 siRNAs (10 µM × 3 siRNAs) or negative control siRNA (30 µM) were mixed with an equal volume of atelogene local use (KOKEN, Tokyo, Japan) and gently incubated for 1 h at 4°C. One day after the removal of the exorbital lacrimal gland, the siRNA was applied to surround the infraorbital lacrimal gland. AQP5 siRNA and control siRNA were used on opposite sides. The next day, mice were anaesthetized with pentobarbital, after which PACAP38 (10–10 M) or saline eye drops were administered. At the 15, 30, 45 and 60 min time points, the tear secretion level was measured using the cotton thread method. The infraorbital lacrimal gland was then removed and the AQP4 and AQP5 levels were checked by real-time PCR and immunostaining.

**Whole-cornea immunostaining.** After euthanasia, the epithelial layer of the cornea was scraped off under a stereoscopic microscope. The eye ball was excised, the skin was then sutured and the animals were kept warm during their recovery from anaesthesia. The tear secretion level was checked before and after surgery. The eye ball was excised, the epithelial layer of the cornea was scraped off under a stereoscopic microscope. The eye ball was excised, the skin was then sutured and the animals were kept warm during their recovery from anaesthesia. The tear secretion level was checked before and after surgery.

**Dot blotting.** PACAP38 or VIP (1 µl of 0.2 to 25 pmol µl–1) was dropped on a nitrocellulose membrane. After drying, the membrane was washed in Tris buffered saline with Tween 20 and blocking buffer, followed by immunoblotting as described above. A primary anti-PACAP antibody (1:4,000; Peninsula Laboratories, Belmont, CA, USA) was used in the dot blotting study.

**MALDI-TOF mass spectrometry of intact PACAP38 in tear samples.** Tear samples were collected from mice by application of sterile filter paper strips (n = 5, Schärger paper), and PACAP38 was measured using MALDI-TOF mass spectrometry. A 1 µl solution of the standard and tear samples were loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by mixing 1.0 µl of each solution with the same volume of a saturated matrix solution, prepared fresh every day by dissolving 3-cyano-4-hydroxycinnamic acid in acetone/0.1% trifluoroacetic acid (1/2, v/v). The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics) operated in the linear mode. Ions were accelerated under delayed acceleration conditions (140 ns) in the positive ion mode with an acceleration voltage of 20,000 kV. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH, Berlin, Germany). External calibration was performed in each case using the average masses of the Bruker Peptide Calibration Standard (r206195, Bruker Daltonics). Protein masses were acquired within a range of 1,000–8,000 m/z. Each spectrum was produced by accumulating data from 800 consecutive laser shots. Bruker FlexControl 2.4 software was used for control of the instrument and Bruker FlexAnalysis 2.4 software for spectrum evaluation.

**Nanospray desorption electrospray ionization Orbitrap MS/MS analyses of PACAP38 in tear samples.** Nanospray desorption electrospray ionization (nano-DESI) was used to acquire MS/MS spectra directly from Schirmer paper containing tears (n = 6). The nano-DESI probe consisted of two fused silica capillaries (ID 50 µm, OD 150 µm, Polymeric Technologies, Molex, Lisle, IL) positioned at an angle to each other. A solvent, consisting of methanol/water (9/1, v/v) with 2% formic acid, was propelled, at 0.5 µl min–1, through the primary capillary, forming a liquid bridge to the secondary capillary. The secondary capillary transported the solvent to the mass spectrometer inlet for nanospray ionization. The filter paper was soaked with 10 µl 0.1% trifluoroacetic acid (99%, Sigma-Aldrich) on a regular glass slide. The glass slide was placed on a motorized x,y,z-stage (Newport Corporation, Irvine, CA, USA) to position the sample under the nano-DESI probe. Material was extracted from the wet surface of the Schirmer paper by the nano-DESI probe and analysed using a QXeactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The instrument mass resolving power was set to 70,000 (m/Δm) and a high voltage of 3 kV was applied to the primary capillary. Selective ion monitoring was set to m/z 648.5 ± 2, corresponding to PACAP38 (z = 7) and tandem mass spectrometry was performed at m/z 648.5 ± 1. Data were processed with FlexAnalysis 2.4 software for spectrum evaluation. The same settings were used for the PACAP38 standard, wild-type mouse samples and PACAP6–38 mouse samples.

**Effect of PACAP on angiogenesis.** The experiments on endothelial cell tube formation were conducted in 24-well dishes using an angiogenesis kit (Kurabo, Okayama, Japan), according to the manufacturer’s instructions. Human umbilical vein endothelial cells and fibroblasts were co-cultured in medium containing vascular endothelial growth factor (final 10 µg/ml) with various concentrations of PACAP38 (10–5, 10–6 M) and PACAP6–38 (10–8, 10–9 M), with the medium exchanged on days 4, 7 and 9. On day 11, the cells were washed and directly fixed in the wells with 70% ice-cold ethanol for 30 min. The fixed cells were serially incubated with 1% bovine serum albumin (BSA) in the buffer, a mouse monoclonal antibody against human CD31, an alkaline phosphatase-conjugated goat anti-mouse IgG, and a nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt from the kit, and then washed and photographed. The images were analysed using Angiogenesis Image Analyzer software (Kurabo) to measure the gross area of the CD31-positive tubes (the area of endothelial tubes) and the length of CD31-positive tubes in culture. Data are shown as a percentage of the area of the endothelial cell tubes in the untreated cultures.

**Systemic infusion of PACAP38.** Systemic infusion of PACAP was done as in our previous study. Briefly, PACAP38 (5 nmol kg–1) or PACAP38 plus PACAP6–38 (50 nmol kg–1) was injected into the jugular vein with vehicle (0.1% BSA in saline) under inhalation anaesthesia with sevoflurane. A PE10 polyethylene tube connected to an Alzet osmotic pump (0.5 µl h–1; DURECT Corporation, Cupertino, CA, USA) was filled with 1% bovine serum albumin (BSA) in the buffer, a mouse monoclonal antibody against rabbit CD31, an alkaline phosphatase-conjugated goat anti-mouse IgG, and a nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt from the kit, and then washed and photographed. The images were analysed using Angiogenesis Image Analyzer software (Kurabo) to measure the gross area of the CD31-positive tubes (the area of endothelial tubes) and the length of CD31-positive tubes in culture.
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Author contributions
T.N. designed and performed the experiments, evaluated the results, and wrote part of the manuscript. H.O. scored the corneal keratinization and performed the molecular biochemistry. T.S. provided valuable expert advice. N.K. performed the eye drop experiment. L.M., I.L., J.F., P.K. and D.R. performed the mass spectrometry experiment and wrote part of the manuscript. S.Y. performed the immunohistochemical experiment. H.N., N.S. and A.B. supplied the experimental material and edited the manuscript. S.S. designed and supervised the experiments and wrote the manuscript.

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