Neuropeptides Released From Trigeminal Neurons Promote the Stratification of Human Corneal Epithelial Cells

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Purpose. To examine the effects of neural cells on the stratification of and junctional protein expression by corneal epithelial cells with a coculture system.

Methods. PC12 cells induced to undergo neuronal differentiation or rat trigeminal nerve cells were cultured together with simian virus 40–transformed human corneal epithelial (HCE) cells on opposite sides of a collagen vitrigel membrane. Stratification of HCE cells was examined by immunofluorescence analysis with antibodies to zonula occludens–1. Expression of junctional proteins in HCE cells was assessed by RT-PCR and immunoblot analyses.

Results. The presence of neural cells (PC12 cells or trigeminal neurons) markedly promoted the stratification of HCE cells as well as increased the amounts of N-cadherin mRNA and protein in these cells. These effects of the neural cells were mimicked by conditioned medium prepared from differentiating PC12 cells or by the neuropeptides substance P and calcitonin gene–related peptide (CGRP). Furthermore, the stimulatory effects of trigeminal neurons on the stratification of and N-cadherin expression by HCE cells were inhibited by antagonists of substance P or of CGRP.

Conclusions. These results suggest that trigeminal neurons play an important role in the differentiation of corneal epithelial cells. Neuropeptides released from these neurons may thus regulate adhesion between corneal epithelial cells and thereby contribute to the establishment and maintenance of corneal structure and function.

Keywords: corneal epithelium, trigeminal ganglion, neuropeptides, stratification, N-cadherin

Interactions among different cell types play a key role in repair of tissue damage in mammals. Interactions between epithelial cells and fibroblasts dominate in mid and late phases of wound healing, when the initial inflammatory response gives way to the synthesis of new tissue components. The cornea is unique in terms of its structure and biological properties. It comprises three major cell types (epithelial cells, stromal keratocytes, and endothelial cells) and receives sensory innervation from the trigeminal ganglion. Sensory nerve endings are confined to the corneal epithelium.[1–3] The sensory nerve bundles enter the peripheral region of the cornea in a radial manner and form a perilimbal ring.[4] Bundles of fibers project to and extend through the corneal stroma, subdivide, penetrate the anterior limiting lamina, and innervate the corneal epithelium, forming a basal epithelial nerve plexus.[2,3] The mechanisms underlying the control of nerve growth in the corneal epithelium remain largely unknown, although wound-induced electric currents have been found to direct nerve regeneration after epithelial wounding.[5]

Intact innervation is necessary for the maintenance of corneal structure and function. Traumatic or surgical lesions of the trigeminal nerve thus result in a form of corneal dystrophy known as neuroparalytic keratitis.[6–8] Destruction of the ophthalmic branch of the trigeminal nerve in mice by stereotactic electrolysis also resulted in an increased frequency of apoptosis and reduced extent of proliferation among corneal epithelial cells.[9] In addition, healing of corneal wounds is retarded by sensory denervation.[8] Corneal nerves maintain a healthy cornea through the release of soluble trophic factors.[10,11] Neuropeptides produced by sensory nerves may contribute to these trophic effects, with substance P (SP), calcitonin gene–related peptide (CGRP), and cholecystokinin having been identified by immunochemical analysis in corneal nerve fibers.[12–14] Trophic interactions between neurons and target cells have been studied extensively both in vivo and in vitro in order to provide insight into the mechanisms of neurogenesis,[15,16] with neurotrophic factors produced by target cells also being thought to play an important role in the survival, differentiation, and maturation of neurons. In particular, there is a bilateral reciprocal relation between epithelial cells and nerves in the cornea, with the former producing neurotrophic factors and the latter producing epitheliotrophic factors.

The corneal epithelium establishes a permeability barrier through the formation of tight junctions (TJs) between adjacent superficial cells.[17–19] The corneal epithelium is a stratified squamous epithelium that undergoes constant renewal with an estimated turnover time of approximately 7 to 10 days in

mammals.[20] It is thus composed of a mitotically active basal cell monolayer and differentiating suprabasal cell layers. The terminally differentiated epithelial cells in the most superficial layer form desmosomes and TJs, which act as a barrier to protect the cornea from environmental insults.[19,21] The molecular mechanisms responsible for the generation and maintenance of corneal epithelial stratification remain poorly understood, however.

We recently established an in vitro model based on the coculture of human corneal epithelial cells and fibroblasts separated by a collagen membrane for the study of epithelial-mesenchymal interactions in the cornea. With this model, we have shown that these two cell types each regulate the function of the other and thereby contribute to corneal homeostasis.[22–27] In particular, we found that the expression of TJ proteins in corneal epithelial cells is upregulated by coculture with corneal fibroblasts in this system.[22] Furthermore, we found that similar coculture of human corneal fibroblasts with the neural cell line PC12 resulted in the upregulation of IL-6 and matrix metalloproteinase–1 in the corneal fibroblasts.[28] These observations thus suggested that interactions among various cell types may play an important role in the maintenance of corneal homeostasis.

We have now examined the role of interactions between corneal epithelial cells and neural cells in corneal homeostasis with the use of a similar coculture system. As neural cells, we examined both differentiated PC12 cells and primary trigeminal nerve cells, with the coculture system, including the latter cells, mimicking the in vivo condition more closely. In particular, we examined the effects of the neural cells on stratification of corneal epithelial cells, a key event in the healing of corneal epithelial wounds.

Methods

Antibodies and Reagents

Rabbit polyclonal antibodies to zonula occludens (ZO)-1 were obtained from Zymed (Carlsbad, CA), those to E-cadherin or to N-cadherin were from Transduction Laboratories (Lexington, KY), and those to neurofilament-L were from Millipore (Temecula, CA). Mouse monoclonal antibodies to α-tubulin were obtained from Sigma-Aldrich (St. Louis, MO), horseradish peroxidase–conjugated secondary antibodies were from Promega (Madison, WI), and Alexa Fluor 488–conjugated secondary antibodies and TOTO-3 iodide were from Molecular Probes (Carlsbad, CA). SP, CGRP, neuropeptide Y (NPY), enkephalin, and GR82334 were obtained from Sigma-Aldrich, and CGRP(8–37) was from PEPTIDE (Osaka, Japan).

PC12 Cell Culture

Rat pheochromocytoma PC12 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% horse serum. The culture medium was changed every 3 days, and the cells were subcultured once a week.

Isolation and Culture of Trigeminal Neurons

The study was performed in accordance with the guidelines of the Committee on Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development, Hiroshima University. It also adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary cultures of sensory neurons were prepared from the trigeminal ganglion of 12- to 14-day-old Wistar rats. The trigeminal ganglion was rapidly excised and enzymatically dissociated in neurobasal medium (Invitrogen, Carlsbad, CA) containing collagenase (3 mg/mL) and the enzyme mixture solution of a Nerve-Cell Culture System (Sumitomo, Tokyo, Japan). The isolated cells were plated on one side of a collagen vitrigel membrane and maintained in neurobasal medium with B27 supplement (Invitrogen), as described below.

Human Corneal Epithelial Cell Culture

Simian virus 40–transformed human corneal epithelial (HCE) cells[29] were obtained from RIKEN Biosource Center (Tsukuba, Japan). The cells were maintained in supplemented hormonal epithelial medium (SHEM), which comprises Dulbecco's modified Eagle's medium (DMEM)–F12 (50:50, vol/vol) supplemented with 15% heat-inactivated FBS, bovine insulin (5 μg/mL), cholera toxin (0.1 μg/mL), recombinant human epidermal growth factor (10 ng/mL), and gentamicin (40 μg/mL).

Coculture of HCE Cells With PC12 Cells or Trigeminal Nerve Cells

PC12 cells or trigeminal neurons were seeded on one side of a collagen vitrigel membrane (diameter of 30 mm; Asahi Technoglass, Tokyo, Japan) in RPMI 1640 medium or in neurobasal medium with B27 supplement, respectively. They were then cultured for 24 hours in a 30-mm culture dish before exposure in the same respective medium to a single dose of recombinant human nerve growth factor (10 ng/mL) (R&D Systems, Minneapolis, MN) for 4 to 5 days. The membrane was then inverted in another dish, and HCE cells (5×10^5) were seeded on the empty side in DMEM supplemented with 10% heat-inactivated FBS. The cells were subjected to air-lift culture for 5 days at 37°C in a humidified incubator containing 5% CO_2. Under these conditions, the neural cells are exposed to serum-free medium in order to maintain their differentiated state. As a control, HCE cells were seeded on a vitrigel membrane without neural cells.

Culture of HCE Cells With Conditioned Medium or Neuropeptides

PC12 cells were seeded on a collagen vitrigel membrane in RPMI 1640 medium, cultured for 24 hours, and then exposed to nerve growth factor (10 ng/mL) for 4 to 5 days in order to induce neurite formation. The medium was then collected, diluted with SHEM, and transferred to support the growth of HCE cells on a collagen vitrigel membrane in air-lift culture. Alternatively, HCE cells were seeded on a collagen vitrigel membrane in SHEM and cultured for 24 hours, after which the medium was changed to serum-free DMEM and the cells were cultured for 24 hours before exposure to neuropeptides (SP, CGRP, NPY, or enkephalin, each at 10 μM) in air-lift culture.

Immunofluorescence Analysis

Cells cultured on a vitrigel membrane were fixed for 15 minutes at room temperature with 3.7% formalin, washed with Ca^{2+}- and Mg^{2+}-free PBS (PBS[–]), permeabilized for 5 minutes with 0.1% Triton X-100 in PBS(–), and incubated for 1 hour at room temperature with 1% bovine serum albumin (BSA) in PBS(–). They were then incubated for 1 hour with antibodies to neurofilament-L or to ZO-1 at a dilution of 1:200 in PBS(–) containing 1% BSA, washed with PBS(–), and incubated for 1 hour with Alexa Fluor 488–conjugated secondary antibodies at

a 1:1000 dilution in PBS(–) containing 1% BSA. The cells were finally examined with a laser confocal microscope (LSM; Carl Zeiss, Jena, Germany). We also prepared paraffin-embedded sections (thickness of 4 μm) of cells on collagen vitrigel membranes. The sections were mounted on silane-treated Aqua glass slides, depleted of paraffin by exposure to xylene, and hydrated with a graded series of ethanol solutions. Antigen retrieval was achieved by treatment with proteinase K (5 mg/mL) for 10 minutes at 37°C. The sections were washed with 0.1% Triton X-100 in PBS(–) and incubated for 1 hour at room temperature with 1% BSA in PBS(–) before immunostaining as described above.

RT-PCR Analysis

Total RNA was isolated as described previously[22] from HCE cells cultured on a collagen vitrigel membrane with the use of an RNeasy kit (Qiagen, Valencia, CA), and portions (0.5 μg) of the RNA were subjected to reverse transcription (RT) and PCR analysis with a One-Step RT-PCR kit based on the Platinum Taq system (Invitrogen). The PCR protocol was designed to maintain amplification in the exponential phase. The sequences of the PCR primers (sense and antisense, respectively) were 5′-TGCCATTACACGGTCCTCTG-3′ and 5′-GGTTCTGCCTCATCATTTCCTC-3′ for ZO-1, 5′-TCCCATCAGCTGCCCAGAAA-3′ and 5′-TGACTCCTGTGTTCCTGTTA-3′ for E-cadherin, 5′-CACTGCTCAGGACCCAGAT-3′ and 5′-TAAGCCGAGTGATGGTCC-3′ for N-cadherin, and 5′-ACCACAGTCCACGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control). The RT and PCR incubations were performed with a GeneAmp PCR System 2400-R (Perkin-Elmer, Foster City, CA). Reverse transcription was performed at 50°C for 30 minutes, and PCR was performed for 25 cycles, with each cycle comprising incubations at 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute. The reaction mixture was then cooled to 4°C. The products of amplification were fractionated by electrophoresis on a 1.5% agarose gel and were stained with ethidium bromide. The intensity of the bands was measured with the use of an image analyzer and Multi Gauge V3.0 software (Fuji Film, Tokyo, Japan), and the values for the abundance of ZO-1, E-cadherin, and N-cadherin mRNAs were normalized by the corresponding value for G3PDH mRNA.

Immunoblot Analysis

Human corneal epithelial cells on a collagen vitrigel membrane were washed twice with PBS and lysed in 300 μL of a solution containing 150 mM NaCl, 2% SDS, 5 mM EDTA, and 20 mM Tris-HCl (pH 7.5), as described previously.[22] Cell lysates were fractionated by SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane and exposed consecutively to primary antibodies and horseradish peroxidase–conjugated secondary antibodies. Immune complexes were detected with enhanced chemiluminescence reagents (GE Healthcare UK, Little Chalfont, UK). Band intensities in the linear range were quantitated by densitometric scanning of film with the use of an image analyzer (Multi Gauge V3 [2] software; Fuji Film).

Statistical Analysis

Quantitative data are presented as means ± SE from three independent experiments and were analyzed with Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

Results

Coculture of HCE Cells and Neural Cells on a Collagen Vitrigel Membrane

We cultured neural cells (PC12 or trigeminal nerve cells) on one side of a collagen vitrigel membrane, exposed the cells to NGF to promote their neuronal differentiation, and then seeded HCE cells on the other side of the membrane and incubated the cocultures for 5 days. Staining of the intact membrane or sections prepared therefrom with antibodies to neurofilament-L (neuronal marker) and with TOTO-3 iodide (for nuclei) revealed that each cell type was restricted to the side of the membrane on which it had been seeded (Fig. 1).

Effects of Neural Cells on the Stratification of HCE Cells

We next investigated the effect of the presence of differentiated PC12 cells on the stratification of HCE cells. Immunostaining with antibodies to the TJ protein ZO-1 revealed that coculture with PC12 cells markedly enhanced the stratification of HCE cells compared with that apparent for HCE cells cultured alone (Fig. 2A). Furthermore, this effect of PC12 cells on HCE cell stratification was mimicked by conditioned medium prepared from cultures of the differentiating neural cells (Fig. 2B). A 1/10 dilution of the conditioned medium was found to be most effective in this regard (data not shown).

We also examined the effects of PC12 cells or their conditioned medium on junctional protein expression in HCE cells cultured on a collagen vitrigel membrane. Immunoblot and RT-PCR analyses revealed that PC12 cells or their conditioned medium induced a significant increase in the expression of N-cadherin at the protein and mRNA levels, without affecting that of E-cadherin or ZO-1, in HCE cells (Fig. 3).

Effects of Neuropeptides on HCE Cell Stratification

Coculture with trigeminal neurons also markedly promoted the stratification of HCE cells (Fig. 4). Trigeminal neurons that project to the cornea produce various neuropeptides that regulate corneal function. We therefore examined the effects of several such peptides (SP, NPY, enkephalin, and CGRP) on the stratification of HCE cells cultured on a collagen vitrigel membrane. We found that SP and CGRP each markedly promoted HCE cell stratification, whereas NPY and enkephalin did not manifest such an effect (Fig. 4). Furthermore, the promotion of HCE cell stratification by coculture with trigeminal neurons was inhibited by the SP antagonist GR82334 and the CGRP antagonist CGRP(8–37) (Fig. 4).

Given that trigeminal neurons also upregulated the expression of N-cadherin, but not that of E-cadherin or ZO-1, at the protein and mRNA levels in HCE cells (Fig. 5), we examined the effects of SP and CGRP on the expression of these junctional proteins in HCE cells cultured on a collagen vitrigel membrane. Immunoblot and RT-PCR analyses revealed that, like trigeminal neurons, both SP and CGRP increased the expression of N-cadherin at the protein and mRNA levels, without affecting that of E-cadherin or ZO-1, in HCE cells (Fig. 5). Furthermore, we found that the upregulation of N-cadherin expression by trigeminal neurons was inhibited by GR82334 and by CGRP(8–37) (Fig. 5). Together, these results thus indicated that SP and CGRP released from trigeminal neurons mediate the stimulatory effects of these neurons on HCE cell stratification and on N-cadherin expression in these cells.

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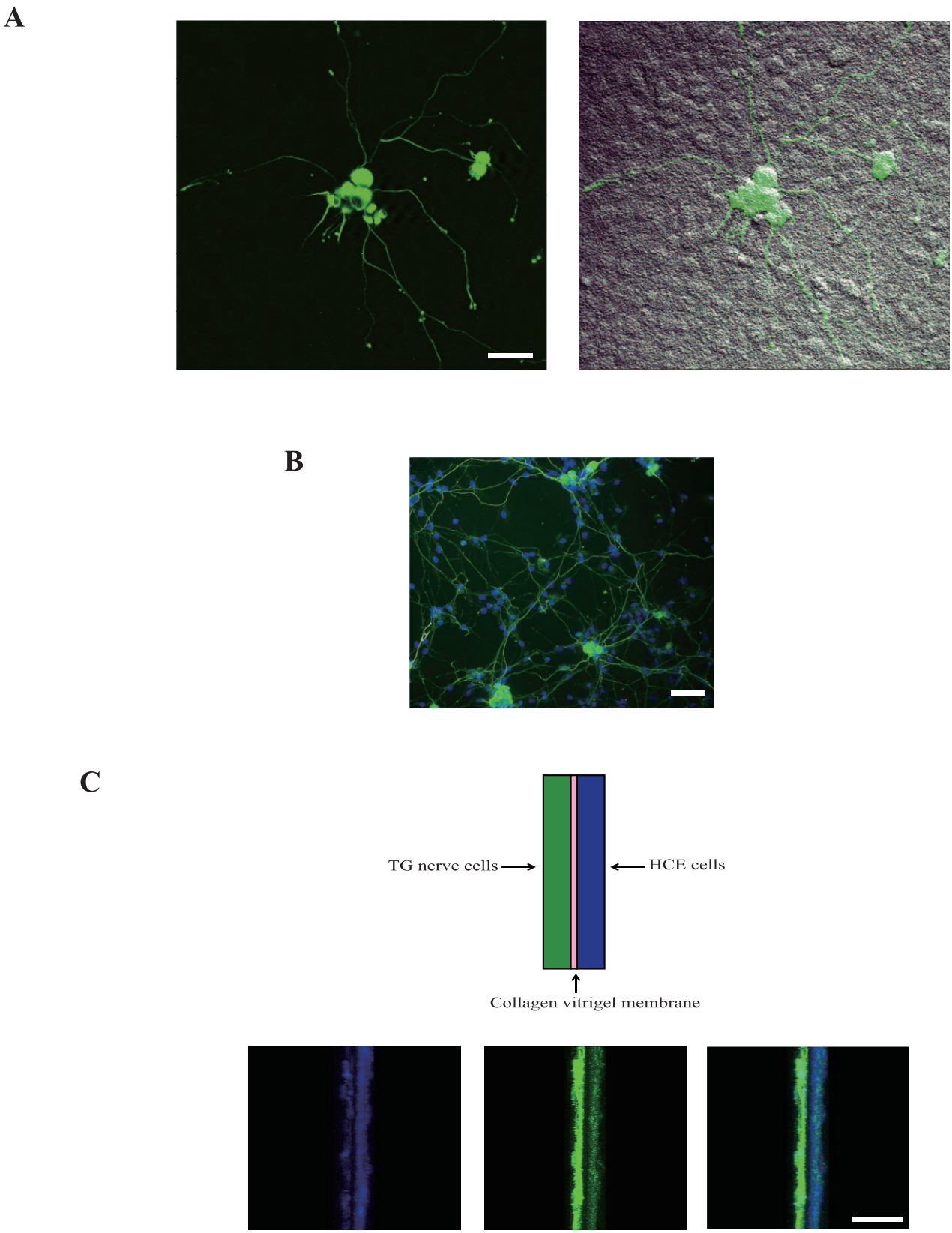

Figure 1. Coculture of HCE cells with differentiated PC12 or trigeminal nerve cells on a collagen vitrigel membrane. (**A**) A collagen vitrigel membrane containing HCE cells on one side and differentiated PC12 cells on the other was subjected to immunofluorescence staining with antibodies to neurofilament-L (*green fluorescence*). A fluorescence image of the side of the membrane containing PC12 cells is shown without (*left*) or with (*right*) a merged phase-contrast image revealing the HCE cells. *Scale bar*: 50 μm. (**B**) Trigeminal neurons were seeded on one side of a collagen vitrigel membrane and cultured in the presence of NGF to promote their differentiation, after which the membrane was inverted and seeded with HCE cells. The side of the membrane seeded with the neurons was subsequently stained with antibodies to neurofilament-L (*green*

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fluorescence) and with TOTO-3 iodide (*blue fluorescence*). *Scale bar*: 50 μm. (**C**) A transverse section of a collagen vitrigel membrane containing HCE cells on one side and trigeminal (TG) neurons on the other was stained with TOTO-3 iodide (*left*) and with antibodies to neurofilament-L (*center*). A merged image is also shown (*right*). *Scale bar*: 50 μm. *Green fluorescence* on the side of the membrane containing HCE cells is likely nonspecific, given that immunoblot analysis of such cocultured HCE cells with the antibodies to neurofilament-L yielded only a weak signal (data not shown).

Discussion

We have shown that the presence of neural cells (differentiated PC12 cells or trigeminal neurons) promoted the stratification of HCE cells cultured on a collagen vitrigel membrane. Coculture with neural cells also increased the expression of the adherens-junction (AJ) protein N-cadherin at both mRNA and protein levels, without affecting that of the AJ protein E-cadherin or the TJ protein ZO-1, in HCE cells grown on a collagen membrane. These effects of the neural cells were recapitulated with conditioned medium prepared from differentiating PC12 cells or by the neuropeptides SP and CGRP. Furthermore, the effects of trigeminal neurons on HCE cell stratification and the expression of N-cadherin were blocked by the SP antagonist GR82334 as well as by the CGRP antagonist CGRP(8–37). Our results thus suggest that neural cells of the cornea might promote the differentiation of corneal epithelial cells as well as the establishment or maintenance of the barrier function of the corneal epithelium, although further studies will be required to verify such a role.

The corneal epithelium is a stratified squamous epithelium, with the basal cells differentiating first into intermediate (wing) cells and then into superficial cells. The differentiation of corneal epithelial cells is accompanied by a switch in the expression of cytokeratin isoforms from K5 and K14 to K3 and K12.[30] In the present study, we used a simian virus 40–transformed HCE cell line as a model for corneal epithelial cells in vivo. We were therefore not able to determine the effects of neural cells on the expression of differentiation markers in stratified corneal epithelial cells in our experimental system. We did show, however, that neural cells promoted HCE cell stratification as well as increased the expression of N-cadherin in these cells. Furthermore, we have identified factors released from the neural cells (the neuropeptides SP and CGRP) that mediate these effects.

Cadherins are calcium-dependent cell adhesion molecules that contribute to AJs and play key roles in embryonic development and in maintenance of normal tissue architecture.[31] The neural isoform N-cadherin is implicated in multiple cellular processes, including cell-cell adhesion, cell differentiation, cell migration and invasion, and signal transduction.[32–36] During gastrulation, cells in the future mesoderm undergo an epithelial-mesenchymal transition that is associated with the upregulation of N-cadherin and the downregulation of E-cadherin. We previously showed that expression of N-cadherin in corneal fibroblasts was upregulated by coculture with HCE cells.[25] The expression of N-cadherin in corneal epithelial cells and fibroblasts thus appears to be regulated by neural cells and corneal epithelial cells, respectively, with such regulation likely

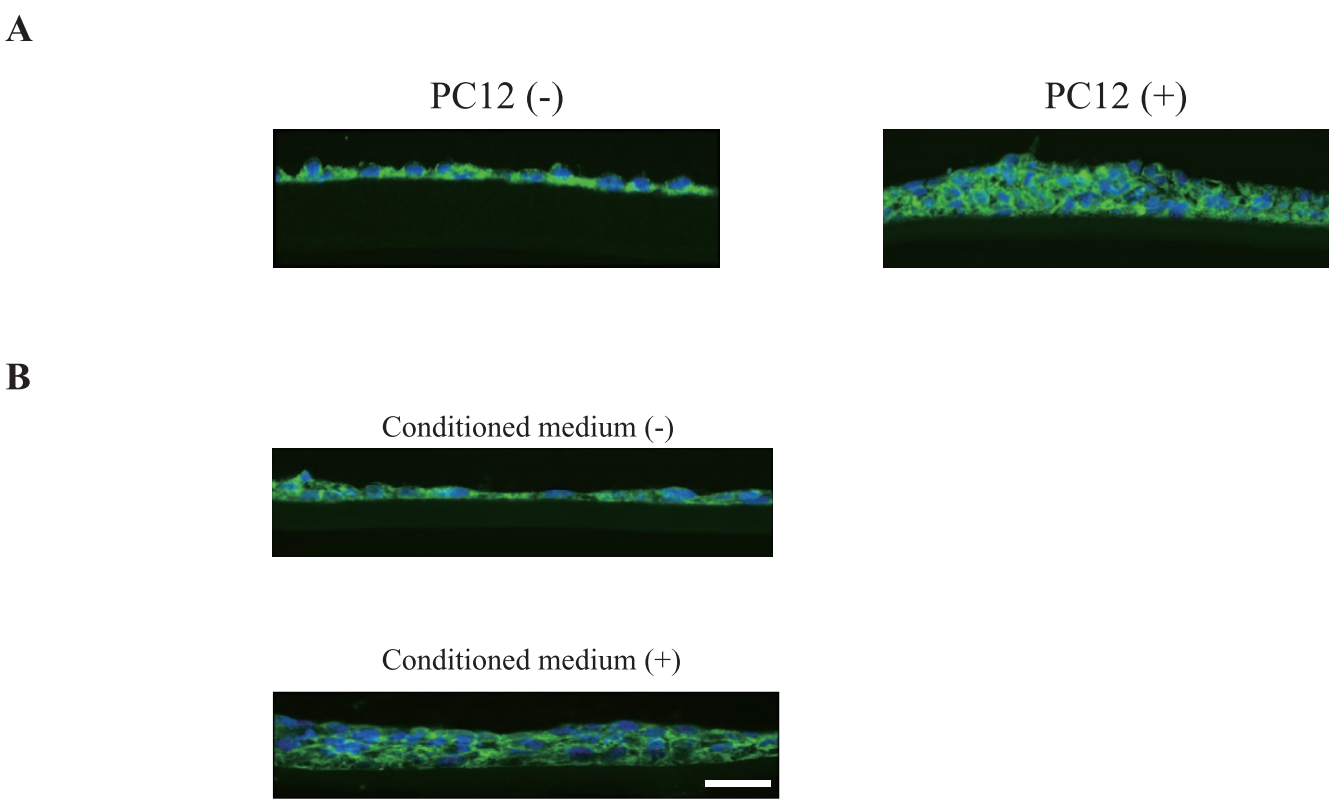

Figure 2. Effect of differentiated PC12 cells on the stratification of HCE cells. (**A**) Sections of collagen vitrigel membranes containing HCE cells cultured for 5 days in the absence (–) or presence (+) of differentiated PC12 cells were subjected to immunostaining with antibodies to ZO-1 (*green fluorescence*) and to staining of nuclei with TOTO-3 iodide (*blue fluorescence*). (**B**) Sections of collagen vitrigel membranes containing HCE cells cultured for 5 days in the absence or presence of conditioned medium prepared from differentiating PC12 cells were subjected to staining as in (**A**). *Scale bars*: 50 μm.

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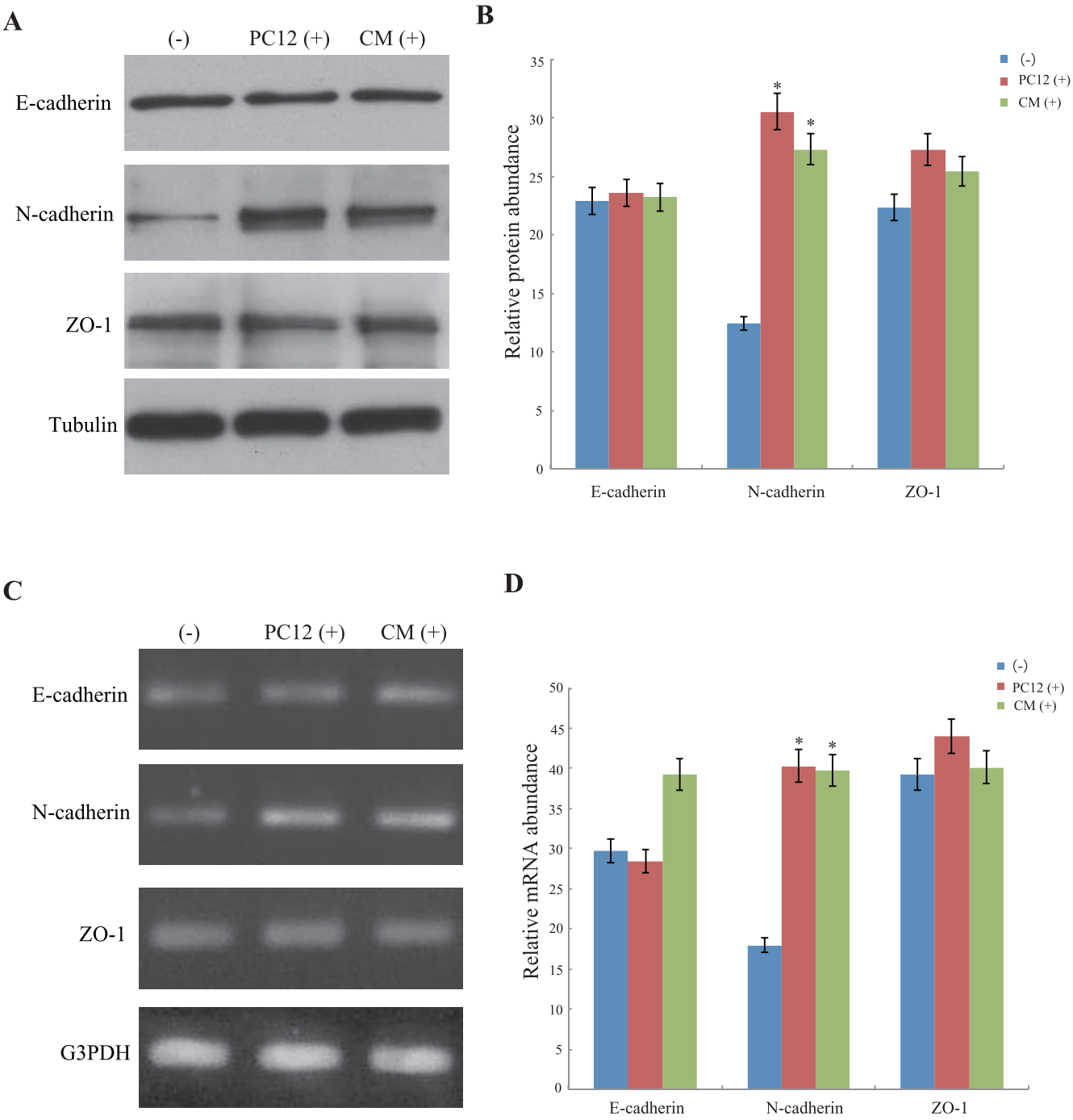
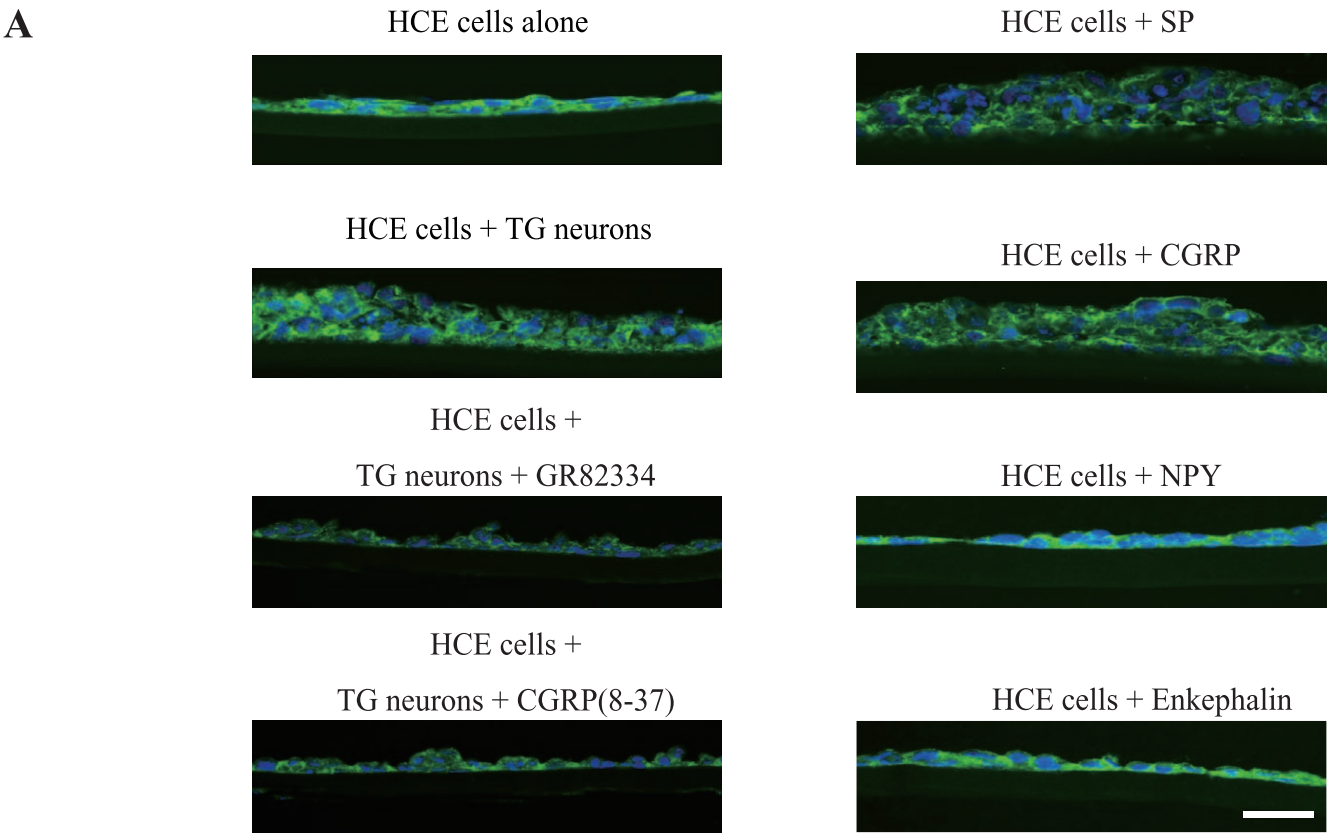


Figure 3. Effects of differentiated PC12 cells or their conditioned medium on junctional protein expression in HCE cells. (**A**) HCE cells cultured on a collagen vitrigel membrane for 5 days in the absence or presence of differentiated PC12 cells or conditioned medium (CM) derived therefrom were lysed and subjected to immunoblot analysis with antibodies to E-cadherin, to N-cadherin, to ZO-1, and to α-tubulin (loading control). (**B**) The abundance of E-cadherin, N-cadherin, and ZO-1 in experiments, similar to that shown in (**A**), was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of α-tubulin. (**C**) Total RNA isolated from HCE cells, cultured as in (**A**), was subjected to RT-PCR analysis of mRNAs for E-cadherin, N-cadherin, ZO-1, and G3PDH (internal standard). (**D**) The abundance of mRNAs for E-cadherin, N-cadherin, and ZO-1 in experiments similar to that in (**C**) was quantified by densitometric scanning of the ethidium bromide–stained gel and normalized by the corresponding amount of G3PDH mRNA. Data in (**B, D**) are means ± SE from three separate experiments. $^*P < 0.05$ (Student's *t*-test) versus the corresponding value for HCE cells incubated in the absence of PC12 cells or conditioned medium.

playing an important role during wound healing and in maintenance of corneal homeostasis. Given that HCE cells cultured with neural cells continued to express E-cadherin despite the upregulation of N-cadherin, the precise and specific contribution of N-cadherin to corneal epithelial cell stratification or differentiation remains to be determined.

Sensory innervation of the cornea by the trigeminal nerve is important for corneal sensation and maintenance of corneal hydration. Corneal nerve dysfunction is responsible for various ocular diseases, such as neurotrophic keratitis and dry eye.[37,38] The precise nature of the interactions between trigeminal nerve cells and corneal cells, including stem cells, epithelial



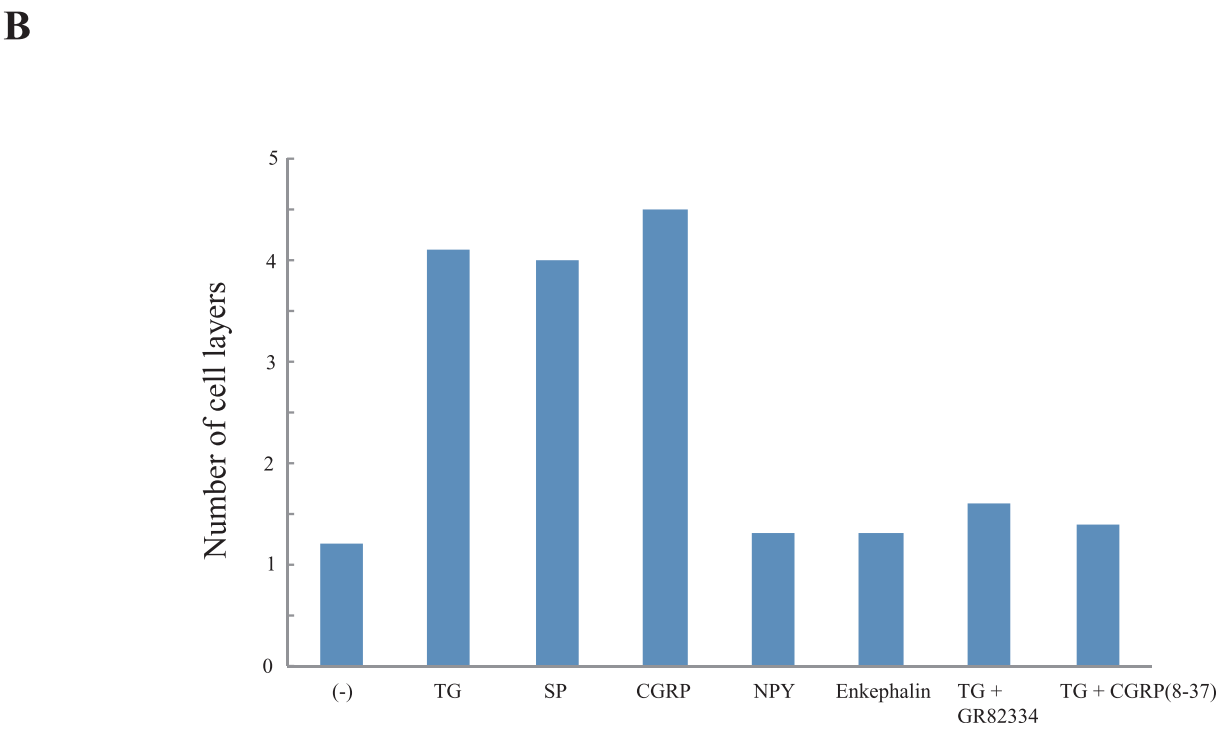


Figure 4. Effects of trigeminal nerve cells or neuropeptides on HCE cell stratification. (**A**) HCE cells cultured on a collagen vitrigel membrane were deprived of serum for 1 day and then incubated in the absence or presence of the indicated neuropeptides (10 μM) for 5 days. Alternatively, collagen vitrigel membranes containing trigeminal neurons on one side were seeded with HCE cells on the other and cultured in the absence or presence of the SP antagonist GR82334 (20 μM) or the CGRP antagonist CGRP(8–37) (20 μM) for 5 days. Sections of all collagen vitrigel membranes were then stained with antibodies to ZO-1 (green fluorescence) and with TOTO-3 iodide (blue fluorescence). *Scale bar*: 50 μm. (**B**) Quantification of HCE cell stratification by determination of the number of HCE cell layers for the experiment shown in (**A**). Stratification was defined as the presence of three or more cell layers.

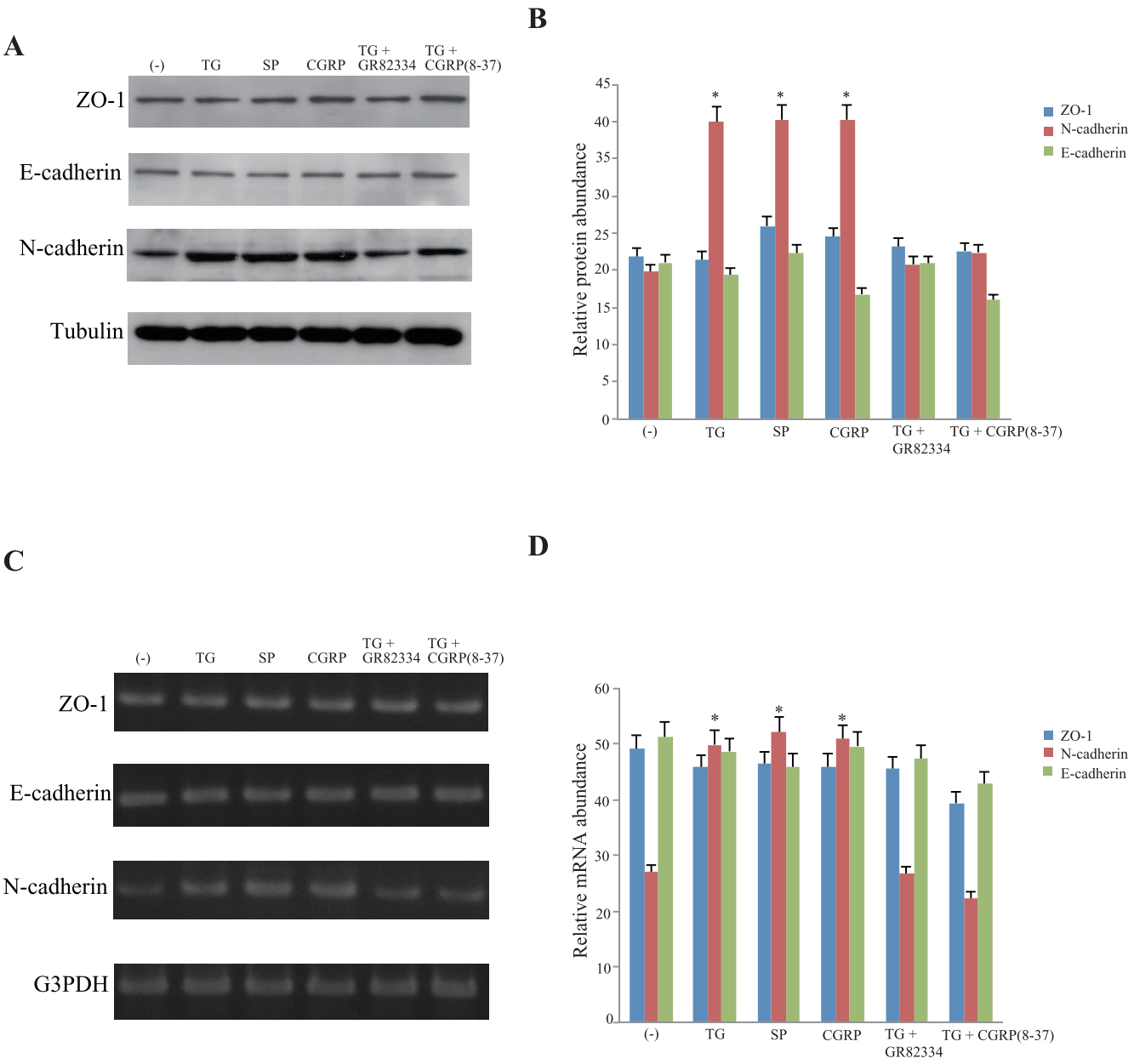


Figure 5. Effects of trigeminal nerve cells or neuropeptides on junctional protein expression in HCE cells. (**A**) HCE cells were cultured on a collagen vitrigel membrane for 5 days in the absence (—) or presence of SP or CGRP (each at 10 μM), or they were cocultured on a collagen vitrigel membrane with trigeminal neurons for 5 days in the absence or presence of GR82334 (20 μM) or CGRP(8–37) (20 μM). The HCE cells were then lysed and subjected to immunoblot analysis with antibodies to ZO-1, to E-cadherin, to N-cadherin, and to α-tubulin. (**B**) The abundance of junctional proteins in experiments similar to that shown in (**A**) was quantified by densitometric scanning of immunoblots and normalized by the corresponding amount of α-tubulin. (**C**) Total RNA isolated from HCE cells cultured as in (**A**) was subjected to RT-PCR analysis of mRNAs for ZO-1, E-cadherin, N-cadherin, and G3PDH. (**D**) The abundance of mRNAs for junctional proteins in experiments similar to that in (**C**) was quantified by densitometric scanning of ethidium bromide–stained gels and normalized by the corresponding amount of G3PDH mRNA. Data in (**B, D**) are means ± SE from three separate experiments. $^*P < 0.05$ (Student's *t*-test) versus the corresponding value for HCE cells cultured alone.

cells, and keratocytes, remains unclear, however. Peptides secreted from trigeminal neurons have been implicated in regulation of the proliferation of corneal cells by the observation that such proliferation is altered after corneal denervation.[39,40]

With the use of a coculture system, we have now shown that trigeminal nerve cells regulate the stratification of corneal epithelial cells as well as the expression of N-cadherin in these cells through the release of SP and CGRP. This model system should prove to be a useful tool for further characterization of the role of interactions between these two cell types in corneal health and disease.

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