Developmental Regulation of Trigeminal TRPA1 by the Cornea

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Purpose. The cornea is densely innervated with nociceptive nerves that detect deleterious stimuli at the ocular surface and transduce these stimuli as sensations of pain. Thus, nociception is a major factor involved in preventing damage to corneal tissues. One class of molecules that is thought to be involved in detecting such stimuli is the transient receptor potential (TRP) family of ion channels. However, little is known about the acquisition of these channels during corneal development. Therefore, the present study examined the developmental acquisition of these receptors and elucidated certain parameters involved in this acquisition.

Methods. Quantitative RT-PCR was used to measure the expression of genes including *TRPA* and *Ret* in vivo. In vitro cocultures between cornea and the ophthalmic lobe of the trigeminal ganglion were used to test interactions between nerves and corneas along with recombinant proteins.

Results. TRPA1 mRNA showed a progressive temporal increase in the ophthalmic lobe of the trigeminal ganglion in vivo during embryonic development. In vitro, TRPA1 expression was significantly increased in the ganglion when cocultured with cornea, compared to ganglia cultured alone. Similarly, the addition of exogenous neurotrophin-3 (NT3) protein to cultured ganglia increased the expression of TRPA1 more than 100-fold. Addition of NT3 and neurturin synergistically increased TRPA1 expression in embryonic day (E)8 ganglia, but this effect was lost at E12. At E8, Ret+ nonpeptidergic neurons are specified in the trigeminal ganglion.

Conclusions. Corneal-derived factors increase TRPA1 expression in trigeminal nonpeptidergic neurons during their embryonic specification.

Keywords: neurotrophin-3, TRPA1, corneal innervation, nociception

The cornea is the anterior-most structure of the vertebrate eye, and as such it is exposed to environmental insults including noxious chemical agents and physical trauma. To respond to and protect against the damage that these insults produce, the cornea is very densely innervated by sensory nerves from the ophthalmic division of the trigeminal ganglion (OTG).[1] This dense corneal innervation is patterned during embryonic development by molecular cues such as Sema3A and Slit2.[2,3] Studies of sensory nerves in other tissues, such as skin, have shown that target tissue-derived factors can also instruct the maturation of sensory neurons into particular subtypes that respond to different stimuli.[4] However, little is known regarding the molecules produced by the cornea that influence the specification of sensory neurons and the mechanisms through which they act.

The sensory nerve fibers within the cornea are unmyelinated Aδ and C fibers that terminate as free nerve endings in the corneal epithelium.[5] These nerve fibers are heterogeneous, with subpopulations responding to mechanical forces, corneal surface cooling, or irritant chemicals.[6] The ability of these nerve fibers to transduce stimuli is critical in modulating the blink reflex, the production of tears, and avoidance behaviors that protect the cornea and maintain proper vision.[6] The nociceptive neurons that innervate the cornea are placode and neural crest derived.[7,8] During embryonic development, nociceptive neurons are initially positive for the TrkA receptor and are dependent on nerve growth factor (NGF), which binds to TrkA, for their survival.[9] As these neurons differentiate and innervate their target tissues, they produce a variety of molecules to detect noxious stimuli, such as transient receptor potential (TRP) channels (discussed below). From the initial TrkA-positive population, two subsets of nociceptive neurons are generated, the peptidergic and nonpeptidergic neuronal lineages.[4] The peptidergic neurons maintain TrkA expression and produce neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P.[4] The nonpeptidergic neurons, which do not produce neuropeptides, will decrease TrkA production and instead begin to express the Ret tyrosine kinase receptor, which is activated by ligands from the glial cell line–derived family (GDNF) of neurotrophic factors.[10,11]

The cornea produces numerous factors that can regulate the growth and differentiation of nerves, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5).[1] These factors have been shown to control a wide range of processes, including neuronal survival, axon growth, neuronal differentiation, and gene regulation,[12] but their effects on nerve subtype specification in the cornea are unclear.

TABLE. Primer List

Gene	NCBI Extension	Forward, 5′–3′	Reverse, 5′–3′
TRPA1, in situ	Gene ID: 420180	AGCTGCCATTTGGTTCCTAAGTCG	ACTGCTTTGAGCACCTACATCC
TRPA1, qRT	Gene ID: 420180	TGGCAGCTCAGAATGGTCATCAGA	GGTGCAAAGCTGTGTTCCCTTCTT
TrkA	Gene ID: 396337	AGGAGAAGACATTGGTGGCTGTGA	ACGCCGTAGAACTTGACGATGTGT
TrkC	Gene ID: 396081	ATGTCACCAGTGAGGACAATGGGT	TCCCATGCACTGCAAAGGCAATAC
Ret	Gene ID: 396107	CACTTTCATCTGTGCCAG	AGTCTTCTCTATCTAGGC
GAPDH	Gene ID: 374193	GGCTGAGAACGGGAAAC	GAGATGATAACACGCTTAGCAC

The OTG neurons innervating the cornea express a wide range of molecules such as purinergic receptors, acid sensing ion channels (ASICs), and the TRP channels mentioned above,[13] that are thought to be involved in detecting damaging noxious stimuli.[1] The TRP channel family consists of 28 members in humans, one of which is TRPA1.[14] TRPA1 is a nonselective cation channel that is present on nociceptive neurons, including trigeminal ganglion (TG) neurons.[15,16] This channel can be activated by cold temperatures (<0°C)[15] and a wide range of damaging compounds, many of which act as lachrymal agents, such as formalin, allyl isothiocyanate,[17–19] and the components of tear gas.[20] In mouse models, injections of molecules that activate TRPA1 induce a range of pain-associated behaviors that TRPA1 knockout animals lack.[18,19]

Given the importance of TRPA1 in detecting noxious stimuli, we examined whether corneal-derived signals are involved in regulating its production in the OTG. We observed that as corneal innervation proceeds, TRPA1 mRNA levels increase in the OTG in vivo. Also, in an in vitro cornea–OTG coculture system, the cornea can increase TRPA1 production in the OTG. Furthermore, we determined that multiple factors can increase TRPA1 levels in the OTG, and that corneal-derived NT3 may be one of the factors responsible for this. However, other factors produced by the cornea, like NGF, for example, are also capable of regulating TRPA1. Lastly, we observed a synergistic increase of TRPA1 in the OTG when NT3 was combined with a ligand (neurturin) that activates the Ret tyrosine kinase receptor[21]—suggesting that NT3 is regulating TRPA1 in a specific subpopulation of nociceptive neurons that have a functional Ret receptor. Taken together, these observations show that NT3 produced by the cornea is involved in regulating the synthesis of TRPA1 in nonpeptidergic OTG neurons.

Materials and Methods

Tissue Isolation

All studies involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fertile eggs (white Leghorn) were obtained from Hyline (Elizabethtown, PA, USA), incubated at 38°C, and staged by the criteria of Hamburger and Hamilton.[22] Corneas were dissected from adjacent tissues and trephined to separate the central cornea region from peripheral cornea to prevent any contamination from the closely associated limbal and scleral tissues. The tissues were stored frozen at –80°C for subsequent RNA isolation (see below).

Trigeminal ganglia were dissected from embryos, and some were fixed in 4% paraformaldehyde (in PBS) overnight at 4°C. For others, OTG were isolated, adherent axons were trimmed away, and the remaining tissue was stored at –80°C for subsequent RNA isolation.

In Situ Hybridization

Messenger RNA was isolated from embryonic day 13 (E13) OTG using Trizol extraction (Invitrogen, Carlsbad, CA, USA). Complementary DNA was then reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Primers for *TRPA1* (Table) were used to amplify products by PCR. The *TRPA1* PCR products were cloned into the pCR4 vector by TOPO-TA cloning (Invitrogen). Cloned sequences were amplified and sequenced using M13 forward and reverse primers to ensure that the proper sequence was cloned, and then antisense RNA probes as well as sense control probes were synthesized from DNA templates using either T3 or T7 polymerase and digoxigenin-labeled nucleotides. Hybridization was performed as previously described.[23]

In Vitro Cornea–OTG Ganglia Coculture

In vitro cocultures of OTG, either with or without cornea, were done as previously described,[2,3] with minor changes. Briefly, embryos were removed and staged. Then, corneas were isolated by trephination, and ganglia were removed by dissection followed by removal of the adherent axons.

For the cultures, type I collagen (3.0 mg/mL; Advance BioMatrix, San Diego, CA, USA) gels were created in four-well chamber slides (BD Falcon, Bedford, MA, USA). Each culture consisted of a cornea placed epithelium side up in the collagen, and two ganglia halves placed 1 to 2 mm on opposite sides of the cornea. The collagen was solidified for 1 hour at 37°C.

The medium consisted of DMEM:Nutrient Mixture F-12 (50:50; Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), antibiotics (100 units penicillin, 100 μg/mL streptomycin; Invitrogen), 5 ng/mL human recombinant epithelial growth factor (Invitrogen), and 5 μg/mL insulin (Sigma-Aldrich Corp., St. Louis, MO, USA) and added to the collagen cocultures.[24] Medium was changed daily, and the cultures were stopped after 96 hours. For RNA isolation, the tissues were dissected from the gels, washed in PBS, and frozen at –80°C. For whole-mount immunofluorescent labeling, the gels were washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS at 4°C for 24 hours.

For experiments conducted on OTG alone, 10 ng/mL NGF, NT3, neurturin (NRTN), fibroblast growth factor-1 (FGF-1), FGF-2 (Sigma-Aldrich Corp.), or GDNF (Abcam, Cambridge, MA, USA) was added to the collagen gel and the culture media.

Whole-Mount Immunofluorescent Labeling

Whole-mount immunofluorescent labeling was performed as previously described with 1 μg/mL anti-beta III tubulin (TuJ-1; R&D Systems, Minneapolis, MN, USA) diluted in 1% blocking buffer.[25]

RNA Isolation and Quantitative RT-PCR

Tissues were thawed on ice, and RNA was extracted with Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, the tissue was homogenized by being passed through a series of needles. Insoluble material was pelleted and

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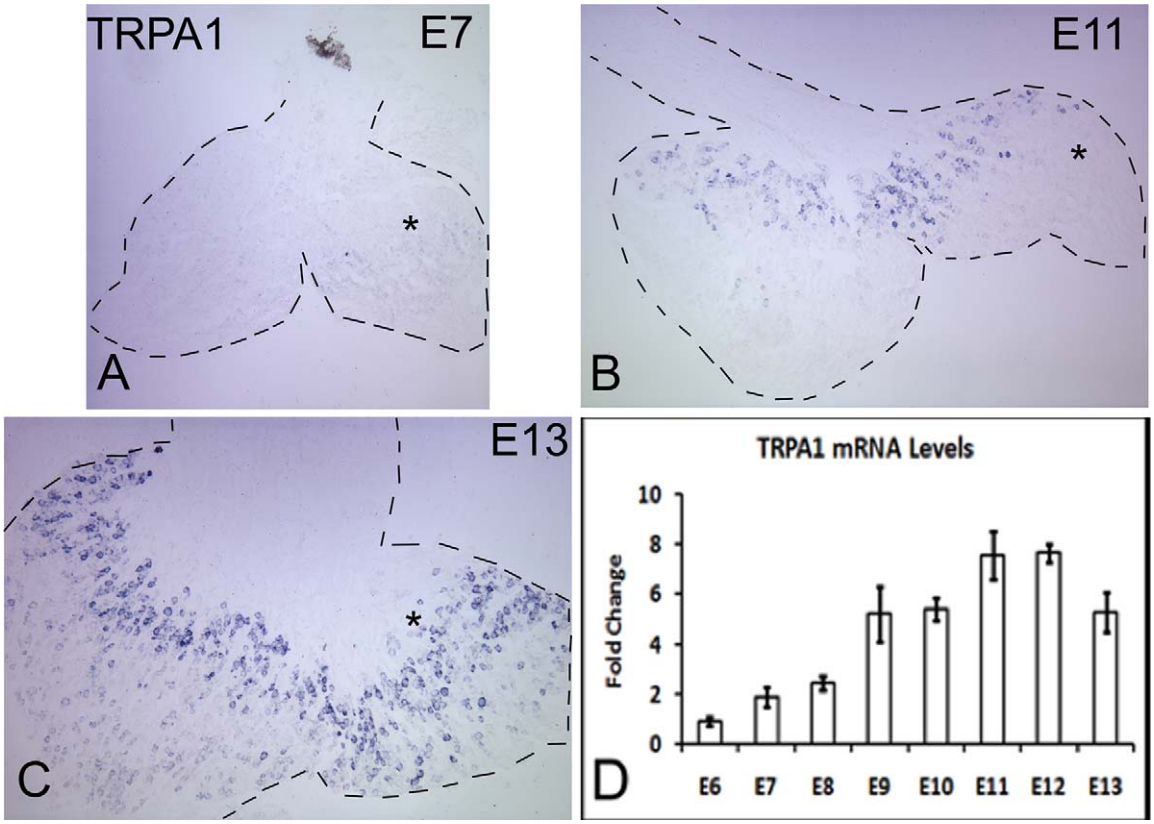

Figure 1. Analysis of TRPA1 mRNA expression profile in the developing OTG. Sections of E7 (**A**), E11 (**B**), and E13 (**C**) TG labeled by in situ hybridization with an antisense probe for TRPA1. Trigeminal ganglion is outlined with a *dotted line*, and the OTG is marked with an *asterisk*. (**D**) Quantitative RT-PCR of TRPA1 in the OTG during development. $n = 5$ independent experiments, 8 OTG/sample/experiment.

chloroform was added to the supernatant, mixed, and centrifuged for phase separation. The aqueous phase was transferred to a clean tube, and the RNA was pelleted by the addition of isopropanol and centrifugation. The RNA pellet was washed with 70% ethanol, air dried, and reconstituted in diethylpyrocarbonate (DEPC)–treated water. RNA concentration was determined by spectrometry, and 1 μg RNA was used to generate cDNA with iScript Reverse Transcription Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. After synthesis, the cDNA was diluted to 15 ng/μL in RNase-free water. Quantitative real-time PCR was performed with 30 ng cDNA and Bio-Rad iQ5 Thermocycler with Bio-Rad SYBR Green Master Mix and the primers in the Table. *GAPDH* was used as the normalizing gene for OTG samples. Fold change was then calculated by the $2^{-\Delta\Delta Ct}$ method.[26]

Statistics

Statistical analyses were performed using 2-way ANOVA with a Bonferroni posttest. Error bars in all figures are ± SEM.

Results

TRPA1 Expression Increases in the OTG of the Developing Chick Embryo

We[25] previously determined that in the embryonic chicken cornea, which is the model we employ in our studies of corneal innervation, all of the nerves originate from the ophthalmic lobe of the TG. Therefore, the assays performed here, using OTG, should apply to all of the corneal nerves.

However, these studies required additional baseline information, such as the temporal relationship(s) between the acquisition of the TRPA1 channels in OTG neurons, and whether such changes are associated with critical events in corneal innervation. To obtain this information, we performed in situ hybridization and quantitative RT-PCR (qRT-PCR) for TRPA1 on embryonic OTG taken at daily intervals encompassing the spatiotemporal events of corneal innervation. These include time points before the OTG-derived axons have entered the corneal stroma (CS) at E6, and when they subsequently penetrate Bowman's layer (E10) to when they extend upward and reach the apical-most corneal epithelial (CE) cells at E13.

Figure 1A shows an in situ micrograph of TRPA1 mRNA in a section through a precorneal-innervation stage (E7) TG, which is circumscribed by a dotted line. In this micrograph the ophthalmic portion of the ganglion is marked with an asterisk.

As can be seen within the OTG, low levels of reactivity for TRPA1 are present in the cell bodies of neurons. Figures 1B and 1C show similar sections at later stages of development (E11 and E13, respectively). By E13, corneal innervation is largely complete, and neuronal cell bodies within the OTG (marked with an asterisk) are now strongly reactive for TRPA1.

While we noted expression of TRPA1 within cell bodies in the maxillary/mandibular lobe of the TG, we restricted our studies to the ophthalmic lobe, as this is the origin of the corneal nerves.

To extend these observations and quantify them, qRT-PCR was performed on RNA isolated from OTG at daily intervals beginning at E6, when the TG has formed but corneal innervation has not yet begun,[27] and proceeding to E12, when nerves have reached the apical layers of the CE (Fig. 1D). Prior

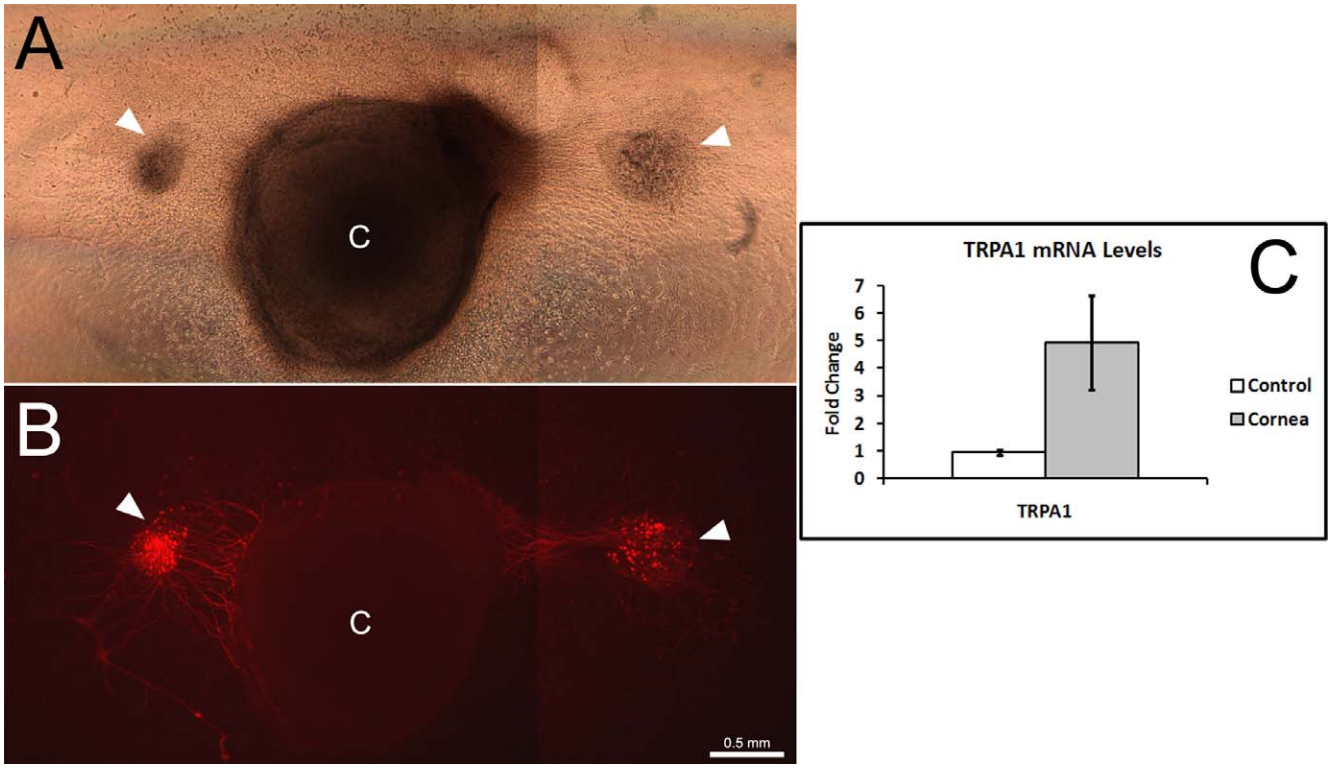

Figure 2. Analysis of cornea–OTG coculture. (**A**) E8 OTG (*arrowheads*) were cultured with E12 cornea (c) for 4 days in type I collagen gels. (**B**) Whole-mount immunofluorescent analysis with the TuJ-1 antibody shows neurites emanating out of the OTG and interacting with the corneal tissues. (**C**) qRT-PCR analysis of OTG dissected from the collagen gels after culturing with E12 cornea shows an increase in TRPA1 compared to control OTG cultured without corneal tissues. $n = 3$ independent experiments, 8 OTG/sample/experiment.

to nerves entering the CS (E6–E7), a slight increase in TRPA1 mRNA occurs. Subsequently, from E8 onward, the TRPA1 mRNA level progressively increases and then levels off (at E11–E12). As these latter times are when innervation of the CE is reaching completion, this is consistent with the involvement of corneal–OTG interactions in regulating the production of TRPA1 mRNA.

TRPA1 Expression Increases in Corneal-OTG Cocultures

While the expression of TRPA1 within the OTG is consistent with changes observed in the time course of corneal innervation, it is unclear if signals from the cornea are directly involved. Because the neurons located within the OTG are responsible for sensory innervation, not only of the cornea but a large portion of the head (i.e., the entire OTG dermatome), it was necessary to determine if signals directly from the cornea could upregulate TRPA1 in OTG neurons. To do this, we employed an organ culture system used previously by ourselves[3] and by others[2] to analyze corneal innervation.

For these experiments, OTG from E8 embryos were cultured either alone (as a control) or with an E12 cornea. Embryonic day 8 was chosen for the ganglia because this is the time in vivo when innervation of the CS is initiated,[25] and it is also when a major increase in TRPA1 mRNA expression occurs (Fig. 2). The corneas were from older embryos (E12), as this is when TRPA1 is maximal (described above). So, if a retrograde signal from the cornea is involved in upregulating the production of TRPA1 in the OTG, it should be active at this time. Also, previous analyses[2,3] showed that younger corneas produce negative regulatory factors (e.g., Slit2 and Semaphorin 3A) that prevent precocious innervation, and that these in organ cultures can prevent the attraction of OTG-derived neurites to the cornea.

For these analyses, we first examined whether this coculture system would be suitable for the proposed studies, and we found this to be the case. In the cocultures, after 4 days, light microscopy (Fig. 2A) showed that extensive cellular outgrowth occurred from both the cornea (C) and OTG (arrowheads). Fluorescence microscopy of these cultures, when labeled for nerves with the TuJ-1 antibody (Fig. 2B), showed that these outgrowths contained extensive neurites, and that these extended from the ganglion to the cornea, which they appeared to contact.

Then, to determine whether this interaction with the cornea resulted in an increase in TRPA1, the OTG were cultured with and without corneas. They were then removed from the cultures and analyzed for TRPA1 mRNA levels by qRT-PCR. As shown in Figure 2C, TRPA1 in the OTG, when cocultured with cornea, was almost 5-fold higher than in ganglia cultured alone, suggesting that one or more corneal-derived factors were responsible for this increase.

NT3 Increases TRPA1 Expression in OTG Neurons

Corneas have been reported to produce a number of different factors that potentially could be involved in regulating neuronal genes.[28] These include NGF,[29] NT3,[30] glial-derived neurotrophic factor (GDNF),[31] FGF-1,[32,33] and FGF-2.[32,34]

To determine which of these factor(s) might be involved in the upregulation of TRPA1, we cultured OTG in medium supplemented individually with each factor, and then measured TRPA1 mRNA by qRT-PCR (Fig. 3). Treatments with FGF-1 and FGF-2 produced slight increases (2.7- and 3.6-fold, respectively). Nerve growth factor and GDNF elicited stronger responses (6.0 and 9.1-

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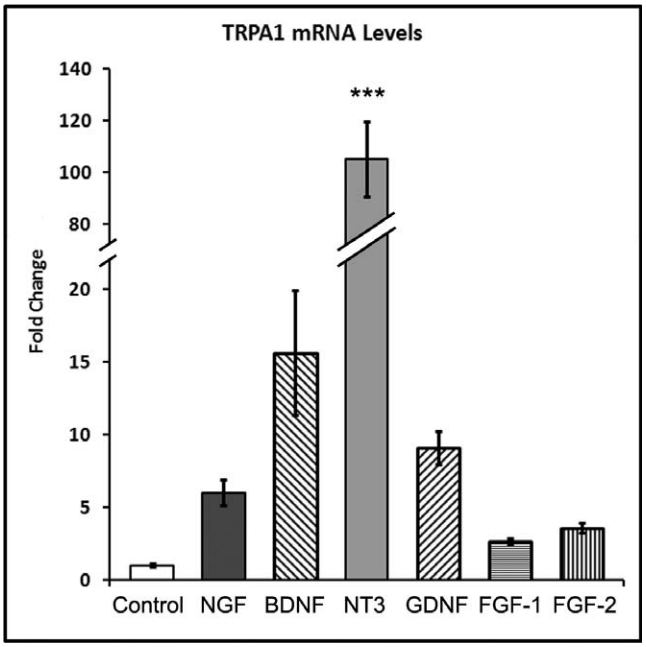

FIGURE 3. Effects of neurotrophic factors on TRPA1 production in the OTG. qRT-PCR analysis of TRPA1 after E8 OTG were cultured either alone or with NGF, NT3, GDNF, FGF-1, or FGF-2 for 4 days ($n = 4$ independent experiments, 8 OTG/sample/experiment). $^{***}P < 0.001$.

fold), which is consistent with previous work showing that neurons having TRPA1 also have receptors for these two factors (i.e., TrkA for NGF and Ret for GDNF[16,35]).

However, by far the greatest stimulation was produced by NT3, which resulted in an increase of more than 100-fold (Fig. 3). This large increase in TRPA1 elicited by NT3 at E8 prompted us to examine this relationship further.

When cultures of older OTG (E10 and E12) were treated with NT3 (Fig. 4, NT3), upregulation of TRPA1 still occurred. However, the response was less than observed with E8 ganglia (i.e., 8.8-fold with E10 [white bar] and 4.8-fold with E12 [gray bar]).

Also for comparison, cultures of E10 and E12 ganglia were treated with NGF, which was another factor that had produced an increase in the expression of TRPA1 in E8 ganglia (Fig. 2, NGF). However, unlike the response observed from different developmental stages of ganglia, treatments by NGF elicited a similar (~5-fold) response from all three ages of ganglia examined (see E8 [Fig. 3, NGF] and E10 and E12 [Fig. 4, NGF]).

Interestingly, levels of NT3 expression at either the mRNA or protein level in the corneal epithelium did significantly change during the time course of corneal innervation (Fig. 5), consistent with previous published descriptions of NT3 expression.[30]

Taken together, these data show that cornea produces multiple factors that are capable of regulating the expression of TRPA1 in the OTG during the period when corneal innervation is taking place. However, there appeared to be an additional level of temporal developmental regulation with NT3 not observed with NGF. Furthermore, this regulation did not appear to involve levels of NT3 expression within the cornea. Therefore, we further investigated this effect of NT3 stimulation on TRPA1 expression.

NT3 and the Ret Ligand Neurturin Synergistically Increase TRPA1 Expression

During development of the dorsal root ganglion (DRG) of mice, nearly all nociceptive neurons are initially TrkA+, and

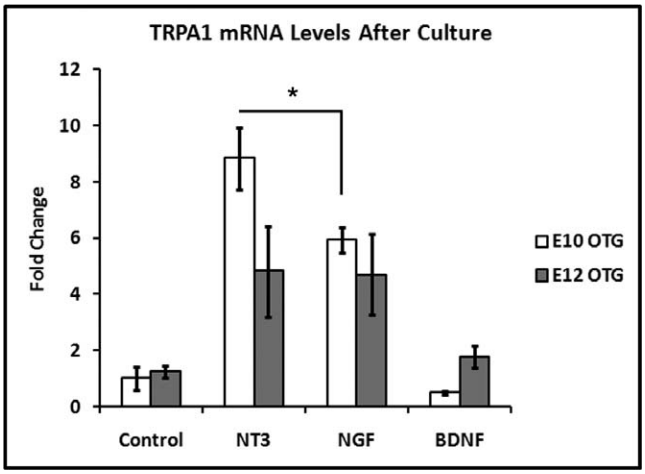

FIGURE 4. Effects of NT3 and NGF on TRPA1 production in E10 and E12 OTG cultures. E10 OTG and E12 OTG were cultured in type I collagen gels either alone or with NT3 or NGF for 4 days and then analyzed for TRPA1 mRNA expression by qRT-PCR ($n = 3$ independent experiments, 8 OTG/sample/experiment). $^{*}P < 0.05$.

subsequently they differentiate into two main categories of sensory neurons—peptidergic, which maintain and increase TrkA, or nonpeptidergic, which lose TrkA and initiate the synthesis of the Ret receptor. The synthesis of the Ret receptor marks the period when lineage specification occurs in nociceptive neurons.[4]

Also, in adult rodent DRG, TRPA1 is expressed primarily by nonpeptidergic Ret+ neurons,[36] and the Ret receptor is activated by members of the GDNF family.[37]

Therefore we examined whether, during development, the decreased levels of NT3-mediated induction of TRPA1 synthesis might be related to the lineage specification of the nociceptive neurons in the OTG.

First, we determined when specification of the peptidergic and nonpeptidergic lineages occurs in the chicken OTG as shown by their levels of Ret (measured from E6 to E12). The data (Fig. 6) indicate that this occurs between E7 and E9, when a greater than 50-fold increase in Ret occurs. Developmentally, this is consistent with the onset of Ret expression in the mouse TG.[38]

The increase in expression of the Ret receptor that occurs in vivo between E7 and E9, when coupled with the large increase in TRPA1 (observed in vitro when E8 OTG are cultured in the presence of NT3), raised the possibility that NT3 was acting directly on neurons that are entering the nonpeptidergic nociceptor lineage.

If so, then early in their specification, before downregulating TrkA (e.g., E8), these neurons should be expressing receptors for both the GDNF family of ligands and neurotrophins including NT3. Therefore, at this time, activating the Ret receptor with a ligand in the presence of NT3 should generate a synergistic increase in TRPA1, as both factors would be acting on the same neuronal population.

Alternatively, an additive response would suggest that the neuronal population responding to NT3 is separate from the Ret-positive one. Furthermore, if NT3 does act on the Ret+ nonpeptidergic lineage, then at later stages, when these neurons have downregulated TrkA, this synergistic response should be abrogated.

To test these possibilities, OTG (E8, E10, and E12) were cultured with either the Ret receptor ligand, NRTN,[21] or with NRTN plus NT3.

When E8 ganglia were cultured with NT3 + NRTN, a highly significant, 250-fold increase in TRPA1 was observed as

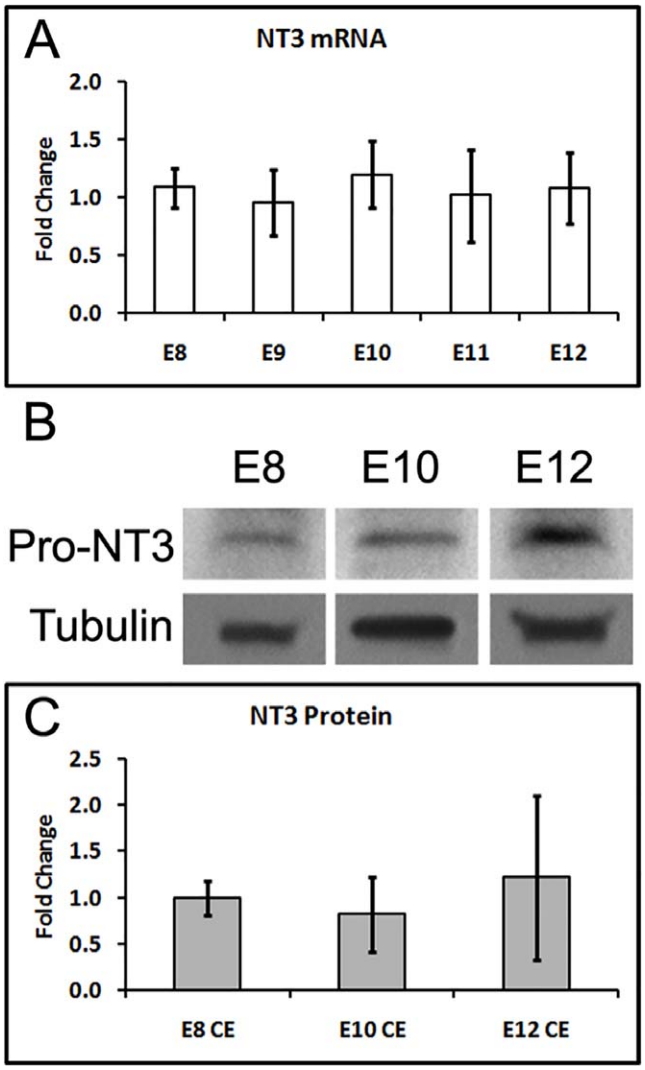

Figure 5. Analysis of NT3 synthesis in the CE. (**A**) Quantitative RT-PCR analysis of NT3 in the CE during the time period of corneal innervation ($n = 5$). (**B**) Western blot of CE tissue from E8, E10, and E12 embryos for NT3 showing detection of Pro-NT3 (~28 kDa) in the CE. Tubulin was used as a loading control. (**C**) Quantification of band intensities of Pro-NT3 normalized to tubulin and E8. $n = 3$ independent experiments, 12 CE/sample/experiment.

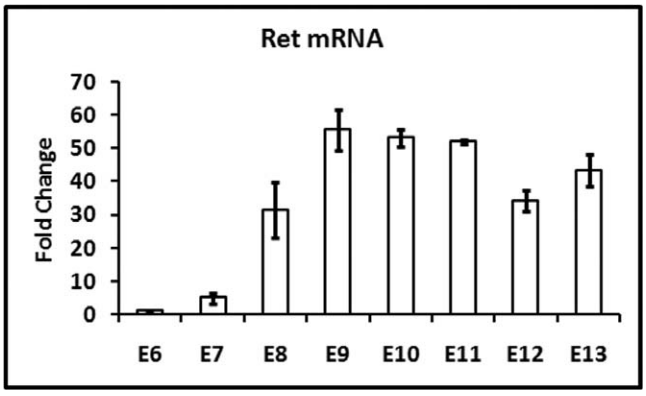

Figure 6. Quantitative RT-PCR analysis of Ret expression in the OTG during development ($n = 4$ independent experiments, 8 OTG/sample/experiment).

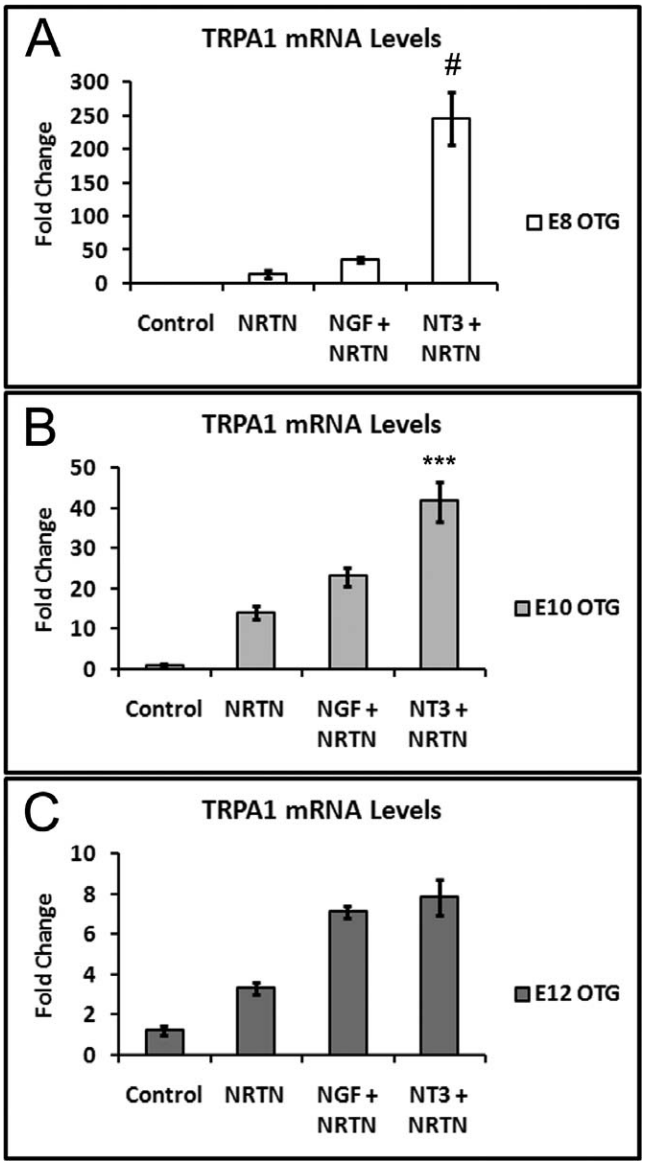

Figure 7. Effects of costimulation of OTG with NT3 and NRTN on TRPA1 production. E8 OTG (**A**), E10 OTG (**B**), and E12 OTG (**C**) were cultured in type I collagen gels for 4 days either alone (Control) or with NRTN or NRTN + NT3, and TRPA1 mRNA levels were analyzed by qRT-PCR ($n = 3$ independent experiments, 8 OTG/sample/experiment). $^{\#}P < 0.0001$, $^{***}P < 0.001$ from all other samples.

compared to the control (Fig. 7A). This response is synergistic, as the total TRPA1 is greater than the 115-fold stimulation that would be predicted for an increase that was additive for each individual component (i.e., 15-fold from the NRTN [Fig. 7A] and 100-fold from the NT3 [Fig. 3]).

When E10 ganglia were cultured with NT3 + NRTN, again a synergistic, statistically significant increase in TRPA1 was observed (42-fold, Fig. 7B), although this response was less than observed at E8. This diminished upregulation is due to a change in responsiveness of the E10 neurons to NT3, as in these cultures the stimulation by NRTN alone was virtually identical to findings for E8 (14-fold [Fig. 7B] versus 15-fold [Fig.7A]).

When the experiment was performed with E12 ganglia, the induction of TRPA1 expression by NT3 + NRTN was diminished even further (to 23-fold) compared to what was seen in the E8 or E10 cultures (Fig. 7C). Furthermore, this

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decrease was due to a diminished response to both NT3 and NRTN. In addition, the response to the costimulation in these E12 cultures was no longer synergistic, as TRPA1 levels were similar to an additive effect for each individual factor.

These results, when taken together, support the hypothesis that NT3 regulates TRPA1 expression in Ret+ neurons as they differentiate into nonpeptidergic nociceptors. Furthermore, the results suggest that as these neurons undergo differentiation, they progressively become refractory to NT3 stimulation, as well as to NRTN, resulting in a diminished increase of TRPA1 in vitro and a plateau of TRPA1 levels in vivo (Fig. 1D).

Discussion

Corneal nerves protect the cornea and preserve vision by transducing damaging stimuli as sensations of pain. However, despite an increased interest in corneal innervation and its role in diseases such as diabetes, keratitis, and dry eye, the mechanisms through which corneal nerves develop and maintain nociception at the ocular surface remain largely unclear.[1] However, it is believed that interactions between the nerves and the cells of the cornea itself are necessary for proper nociception and corneal protection. Previous work has shown that in other tissues, for example, skin, both the intrinsic neuronal development and target-derived factors are necessary for the proper development of sensory nociception.[4] Functionally, an important part of this process is the neuronal expression of membrane proteins that form channels responsible for nociception. One of these channels, which detects potentially damaging chemical irritants (e.g., the components of tear gas[20]) and is present on corneal nerves, is TRPA1.[16]

In the present study we have examined the developmental appearance of TRPA1 in OTG neurons and determined that corneal-derived factors, including both NGF and NT3, are likely involved in regulating its expression in the nerves innervating the cornea. Furthermore, this work demonstrated that during the process of nociceptor specification, NT3 and activation of the Ret receptor with NRTN, a member of the GNDF family, synergistically increased TRPA1 expression. To our knowledge, this is the first study to describe a relationship between NT3 and TRPA1 regulation.

Much of this work relied on a corneal–OTG three-dimensional coculture model system, which has been used previously to study corneal–nerve interactions.[2,3] As the coculture system allowed for the direct contact of neurites with the cornea, regulatory factors that are either secreted or membrane anchored could be involved. While we examined secreted factors such as NT3, our data do not rule out the possibility that membrane-anchored factors may also be involved in regulating TRPA1 expression.

Another caveat in using this culture system is that all the neurons within the ganglion are potentially able to respond to the cues from the cornea, whereas in vivo, only a fraction of these neurons[13,39] would innervate the cornea, with the rest innervating other areas within the dermatomal distribution of the OTG. Therefore, it is possible that these in vitro interactions may alter the expression levels of TRPA1. However, this system more closely mirrors the in vivo setting than do monolayer cultures,[40] and a comparison of the in vivo expression of TRPA1 with the in vitro data shows very similar levels. Furthermore, the timing of changes within these levels is consistent as well, suggesting that these experiments accurately reflect normal development.

Our data suggest that these changes in TRPA1 are regulated both by the cornea, through the release of factors like NT3 and NGF, and by the developmental specification of nociceptor subtypes within OTG neuronal populations that are dependent on a switch between TrkA and Ret.

Neurotrophin-3 is produced by the CE throughout development[30] (data not shown), yet TRPA1 levels in the OTG do not reach a maximum until E12. This raises the question of whether the changes in NT3 responsiveness are a result of OTG neuron development or corneal production of a developmentally regulated inhibitory factor. Our data show the former, but we cannot rule out the latter, as older OTG (E10 and E12) had a dramatic reduction in their response to NT3 when compared to E8 OTG, suggesting that a mechanism intrinsic in the neurons prevents the continued synthesis of TRPA1 in the presence of NT3.

One potential mechanism for the change in NT3 responsiveness would be the downregulation of NT3 receptors, TrkA and TrkC.[9] However, TRPA1 is not found on neurons that are positive for TrkC,[16] suggesting that NT3 is not signaling through this receptor to control TRPA1 levels. Nevertheless, TRPA1 is found on neurons that express TrkA.[16] TrkA is the high-affinity receptor for NGF,[9] and our data indicate that NGF can increase TRPA1 in the OTG, although not to the levels observed with NT3. It has also been shown that NT3 can signal through TrkA to support cultures of neurons isolated from embryonic mouse trigeminal ganglia at E13 but not at E18.[41] Coincident with this change in the ability of NT3 to support mouse trigeminal neurons in culture is the temporal appearance of the Ret tyrosine kinase receptor. Ret is first produced by the TG at E13.5 to E14.5 in the mouse[38] and marks the beginning of the developmental specification of nociceptors.

The nonpeptidergic lineage of nociceptive neurons initially arises from TrkA-positive neurons, but during their specification they become dependent on signaling by the GDNF family of ligands through the Ret receptor. This switch from TrkA signaling to Ret signaling is necessary not only for their survival, but also their differentiation, as mice that lack the Ret receptor in their sensory neurons fail to express TRPA1.[35] While no significant changes in TrkA mRNA levels were observed in the OTG between E8 and E12 (data not shown), it remains possible that neuronal subtypes within the ganglia could be altering their TrkA levels. However, future work is needed to determine if TrkA is specifically downregulated in the neurons responding to NT3 with an increase in TRPA1 production.

Our data suggest a model in which NT3 signals through the TrkA receptor at the time when nociceptors are first being specified, when the nonpeptidergic neurons are beginning to express Ret but still utilize TrkA signaling. This specification occurs in the OTG at E8, as determined by qRT-PCR for Ret (see Results), and leads to a synergistic increase in TRPA1 expression at E8 when OTG are cultured with physiologic levels of NT3 and NRTN. This synergistic effect decreases at E10 and is then further decreased in older OTG cultures at E12, consistent with the specification of Ret+ nociceptors and the downregulation of TrkA signaling in these neurons. However, further studies are needed to determine the role of other corneal-derived factors in regulating TRPA1 expression in the nociceptive neuronal populations within the OTG during corneal innervation.

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