Thyroxine Increases the Rate but Does Not Alter the Pattern of Innervation during Embryonic Chick Corneal Development

Abigail H. Conrad, Jessica M. Strafuss, Maria D. Wittman, Sabrina Conway, and Gary W. Conrad

PURPOSE. Embryonic chick corneal nerves reach limbal mesenchyme by embryonic day (E)5, encircle the cornea in several days, then defasciculate into the stroma simultaneously from all sides, while extracellular keratan sulfate proteoglycan (KSPG) accumulates from posterior to anterior stroma. Precocious thyroxine (T4)-induced increases in corneal thinning/transparency are blocked by 2-thiouracil (2-TU) inhibition of T3 synthesis. The hypothesis for this study was that precocious T4 exposure increases corneal innervation similarly.

METHODS. E8 embryos received T4, 2-TU, T4+2-TU, or buffer; corneas were harvested on E12. Corneal nerves were stained with neuronal β-tubulin-specific TuJ1 antibody or chick nerve-specific CN antibody. Corneal thickness was determined from cryostat sections, and mRNA expression was measured by real-time PCR.

RESULTS. Nerves avoided the cornea until E9, then entered the anterior stroma, extended toward and reached the cornea center by E14, and never invaded posterior stroma. E7 to E18 corneal expressions of nerve growth factor and neurotrophin-3 genes were unchanged; receptor gene expressions rose. E7 to E12 semaphorin 3A and 3F and ephrin A2 and A5 expressions did not change significantly; semaphorin and ephrin/eph expressions increased from E9 to E18. E8 T4 administration increased nerve extension by E11, but did not alter circumferential penetration, anterior-only penetration, or neurotrophin expressions. 2-TU prevented T4-induced precocious corneal thinning, but augmented T4 nerve stimulation.

CONCLUSIONS. No changes in corneal neurotrophin or nerve pathfinding gene expressions accompany corneal transition to nerve growth cone permissiveness. T4 increases corneal nerve penetration rates by a non-T3-dependent mechanism. Results are consistent with possible roles for corneal KSPGs in regulating corneal nerve growth. (Invest Ophtalmol Vis Sci. 2008; 49:139–153) DOI:10.1167/iovs.07-0800

The cornea is one of the most highly innervated tissues on the body's surface. Embryonic chick corneal nerves are derived from neural crest (NC) progenitors, whose cell bodies occupy the ophthalmic lobe of the trigeminal ganglion (TG). 1 If ectodermal placode-derived nerves are prevented from copopulating the TG ophthalmic lobe, corneal innervation is reduced and disrupted,1 suggesting that TG ectodermal placode neurons act as pathfinder axons2 for TG NC neurons in reaching pericocular tissue. As early as Hamburger-Hamilton stages (HH)13 to 15,3 chick TG ophthalmic lobe placode-derived neurons express more transmembrane EphA5 receptor tyrosine kinase, an axon guidance molecule for growing axons,4 than do postmitotic neurons in the maxillary and mandibular lobes of the TG. Thus, their ophthalmic tract pathfinding axonal growth cones guide TG ophthalmic lobe axons toward head ophthalmic mesenchyme by avoiding maxillary and mandibular mesenchyme cells, which express high levels of the Eph receptor ligands ephrin A2 and A5 at this time.5 Bec6 observed that corneal nerve axon fascicles have reached the ventro-temporal pericocular mesenchyme by E6, but subdivide and extend dorsally and ventrally to form a limbal nerve ring around the cornea without entering it until E10. The limbal nerve ring then further defasciculates, and nerves invade the corneal stroma from all around the cornea simultaneously, extend toward the center of the cornea, penetrate from the stroma into the epithelium by E14, and reach the center by E16.6,7

During this time, the chick cornea is also initiating its transition from translucence to transparency. Chick corneal epithelium separates from the lens by E3, and deposits a primary stroma consisting primarily of collagen fibrils on its posterior surface.8 From E3 to E5 non-neuronal NC-derived cells migrate across the posterior surface of the primary stroma, forming corneal endothelium.9 From E5 to E12 other non-neuronal NC-derived pericocular mesenchyme cells invade the cornea primary stroma,9 differentiate into corneal keratocytes, and synthesize and secrete collagens and keratan sulfate- and chondroitin/dermatan sulfate-proteoglycans (KSPGs and CS/DSPGs) into corneal extracellular matrix (ECM) to form a complex thickening secondary corneal stroma.10–12 On E9 extracellular KSPG begins to accumulate in the posterior cornea stroma and endothelium, and this extracellular accumulation of KSPG progresses anteriorly in a wave across the cornea until the entire stroma is filled with extracellular KSPG by E16.13 The cornea reaches maximum thickness/opacity by E12, then undergoes compaction and achieves maximum transparency by E20.13 During chick development, maternally deposited thyroxine (T4) and 3,5,3'-triiodothyronine (T3) are used by the embryo by E6.14 The developing thyroid gland begins to synthesize T4 and T3 by E9,15 and plasma and ocular levels of T4 and T3 begin to rise by E10.14 Precocious in ovo treatment of E7 to E12 chick embryos for 2 to 3 days with T4 causes their corneas to undergo dehydration,16 decrease their thickness,17 increase their concentrations of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), an intermediate in KS and CS/DS biosynthesis,18 and become more transparent than controls. In contrast, corneal thinning is prevented by in ovo treatment with 2-thiouracil (2-TU). We recently showed that precocious T4 treatment of chick embryos increases corneal expressions of mRNAs for nuclear thyroxine hormone receptor beta and carboxic anhydrase II, an enzyme that generates bicarbonate ions,17 suggesting that generation and movement of bicarbonate
| Table 1. PCR Primers for Nerve Growth Factors, Guidance Regulators, and Thyroxine Integrin Receptors

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Primers</th>
</tr>
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<tr>
<td><strong>Nerve Growth Factors, Receptors, and Schwann Cell Regulators</strong></td>
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<tr>
<td>NRG1</td>
<td>Glial fibrillary acidic protein, GI:50760636 F: 5</td>
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<td>TAC1</td>
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<td>R: 5'GACAAGACCGAGGAGGAGC3'</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein, GI:50760636 F: 5</td>
<td>F: 5'CCTCTGCTGCTGAGACG3'</td>
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<td>Neurophin 2, GI:45383569</td>
<td>F: 5'GCCACACACATGGTGGC3'</td>
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ions may regulate corneal hydration and transparency, and thus that increasing concentrations of T₄/T₃ in the eye may contribute to normal transparency development in the avian eye.

Observations of early nerve exclusion from the cornea suggest that nerve growth cone-repulsive signals are released from the early developing cornea and that at a critical time something changes in the cornea that either terminates the nonpermissive signals or overrides them with nerve growth cone permissive signals. The nature of these signals or other changes in the cornea that regulate nerve growth cone permissiveness are not well understood. Nerve growth factor beta (NGFB), brain-derived nerve factor (BDNF), and neurophin-3 (NTF3) act as positive guidance cues for TG axons. Disturbances in the expression of the growth cone chemorepellant guidance molecules, semaphorins and their neuropilin and plexin receptors, disorganize peripheral TG axon tracts. Because the transition period for corneal nerve penetration coincides with increases in T₄ and T₃ in the eye, we hypothesized that precocious exposure of chick embryos to T₄ alters corneal innervation in ways that elucidate normal mechanisms for regulating corneal innervation.

### Table 1. (continued) PCR Primers for Nerve Growth Factors, Guidance Regulators, and Thyroxine Integrin Receptors

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<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Primers</th>
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| PLXNA2 | Plexin A2, GI: 118102444 | F: 5′-CACAAGAGGAGCACTACC-3′  
R: 5′-AAATATCCGTCCCCACATCG-3′ |
| PLXNA4 | Plexin A4, GI:118081940 | F: 5′-ATGTGAAGTGGCATGCCGAC-3′  
R: 5′-CCAGTGAGGTTAGGAAGG-3′ |
| PLXNB1 | Plexin B1, GI: 118096955 | F: 5′-TTTCCACCGCAGCTACG-3′  
R: 5′-TCTCCATACAGGTAAGG-3′ |
| PLXNB2 | Plexin B2, GI:118082194 | F: 5′-CACGTGCTCTCTGTTAGC-3′  
R: 5′-ATCCTTTTGTACCTACATCC-3′ |
| PLXNC1 | Plexin C1, GI:118082506 | F: 5′-TGATTAGTTGCTTTTGATGG-3′  
R: 5′-TGGGAGGATGGTAAGACC-3′ |
| PLXND1 | Plexin D1, GI:118097128 | F: 5′-TTATGATCGACCTGGAGAC-3′  
R: 5′-TGATGCGAAGCTGGAAGG-3′ |

#### Eph receptors and ephrins

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<th>Symbol</th>
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<th>Primers</th>
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| EPHA1 | EPH receptor A1, GI:45383435 | F: 5′-AGAGTTCTACGGTACCTCTCG-3′  
R: 5′-GCCCTGGAAGTGGGAGAC-3′ |
| EPHA3 | EPH receptor A3, GI:454809 | F: 5′-AGTGAAATAGCCAGCTGTATTG-3′  
R: 5′-GATCCGCAAGATCCTCAATCTG-3′ |
| EPHA4 | EPH receptor A4, GI:45382184 | F: 5′-GTGTCGATGCTGGAATGAC-3′  
R: 5′-CCCACGCTGAGGAAGAGG-3′ |
| EPHA5 | EPH receptor A5, GI:47087152 | F: 5′-AGAGAAGTACACCGCACTCAGT-3′  
R: 5′-TTCTCCAGCCATATACCCAG-3′ |
| EPHA6 | EPH receptor A6, GI: 50729751 | F: 5′-CAGTCGATCGAATGAGAGG-3′  
R: 5′-ACTGTTGGGCTCCTCAATC-3′ |
| EPHA7 | EPH receptor A7, GI:45384173 | F: 5′-CTCAGGAGGTTAATACGCTCTC-3′  
R: 5′-GATCCGCAAGATCCTCAATCTG-3′ |
| EPHA8 | EPH receptor A8, GI:50759486 | F: 5′-AGCTCTCGGTCTCTCCTTG-3′  
R: 5′-ACGTAGGTCCCAGTGGAAC-3′ |
| EPHA9 | EPH receptor A9, GI:13517099 | F: 5′-AGAGTTCTACGGTACCTCTCG-3′  
R: 5′-GCCCTGGAAGTGGGAGAC-3′ |
| EPHB1 | EPH receptor B1, GI:50752165 | F: 5′-TTATGAGATTCGAAGTCCCGAT-3′  
R: 5′-GATCCGCAAGATCCTCAATCTG-3′ |
| EPHB2 | EPH receptor B2, GI:46395488 | F: 5′-GCCCTGCTCTCGCCACTG-3′  
R: 5′-ATGCTATAACTCACTCAATCTG-3′ |
| EPHB3 | EPH receptor B3, GI:50752376 | F: 5′-TTTGGTTAATGCACTCCTCCAGATG-3′  
R: 5′-CGGAGATGCCAGAAGCTGTC-3′ |
| EPHB6 | EPH receptor B6, GI:52138668 | F: 5′-CCTCGCTGTCGCCCTG-3′  
R: 5′-CCTCGCTGTCGCCCTG-3′ |
| EFNA2 | Ephrin-A2, GI:45384481 | F: 5′-ATCCTCAGCAGCTGTCGAC-3′  
R: 5′-CCTCGCTGTCGCCCTG-3′ |
| EFNA5 | Ephrin-A5, GI:45383987 | F: 5′-TTGAGACGACAGATGAGCG-3′  
R: 5′-GCCGACGAGAACAATAGTGTG-3′ |
| EFNA6 | Ephrin-A6, GI:12656587 | F: 5′-TTTCATAGCCTGCTGTC-3′  
R: 5′-GTGGTCTGTCGATGGACACTCT-3′ |
| EFNB1 | Ephrin-B1, GI:45384291 | F: 5′-ATCCTCAGCAGCTGTCGAC-3′  
R: 5′-GCCGACGAGAACAATAGTGTG-3′ |
| EFNB2 | Ephrin-B2, GI:45382248 | F: 5′-CTGGTCCTCGAGAGTAC-3′  
R: 5′-CTGGTCCTCGAGAGTAC-3′ |

#### Thyroxine Integrin Receptors

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<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Primers</th>
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| ITGAV | Integrin aV, GI:4582132 | F: 5′-GATCCGAAGTTCCTCCTCCTT-3′  
R: 5′-GCTGTCGAAATGGCAGATGTC-3′ |
| ITGB3 | Integrin b3, GI:474038 | F: 5′-ACGTCTCCTCGTCCGCT-3′  
R: 5′-GCGTAGTCCACGGTACGTC-3′ |
Materials and Methods

Embryo Culture and Cornea Isolation

Fertile White Leghorn chicken eggs were transferred to a 38°C incubator on E0 for incubation. Corneas from embryos of the desired age and/or treatment protocol were dissected in sterile saline G (Sal G: 1.37 mM NaCl, 2.7 mM KCl, 4.5 mM NaHPO4, 1.4 mM KH2PO4, 6.1 mM glucose, 0.6 mM MgSO4, and 0.1 mM CaCl2, pH 7.4). For RNA isolation, noncorneal tissue was trimmed away, and corneas were quick frozen in liquid nitrogen and stored at −70°C until used. These corneas include both peripheral and central corneal tissue, as defined by Müller et al.27

In Ovo Administration of T4 and 2-Thiouracil

Eggs injected with T4 or SalG alone were handled as described previously,29 except that the eggs were injected on E8. For injections of T4+2-TU or 2-TU alone, the eggs were turned on their sides on E8, a small piece of egg shell plus inner shell membrane was removed, and solutions were deposited onto the chorioallantoic membrane. Nontreatment controls received 150 μL SalG containing 5 μg streptomycin and 5 units penicillin (SalGp/s). T4 controls received 100 μL SalGp/s containing 5 μg T4 and 50 μL SalGp/s. T4+2-TU-treated eggs received 100 μL SalGp/s containing 5 μg T4 plus 50 μL SalGp/s containing 10 mg 2-TU, and 2-TU-treated eggs received 100 μL SalGp/s plus 50 μL SalGp/s containing 10 mg 2-TU. Egg shell holes were sealed with transparent tape, and eggs were incubated on their sides at 40% to 45% relative humidity, 38°C, for 3 days. Embryonic corneas were then removed and prepared for nerve staining or RNA isolation.

Corneal Nerve Staining

Whole cornea nerve staining was performed as described previously,29 with the following changes: The primary antibody was anti-neuronal class III β-tubulin (TuJ1; CRP Inc., Denver, PA), diluted 1:1000; the secondary antibody was horseradish peroxidase–conjugated goat anti-mouse IgG (H+L; Invitrogen, Carlsbad, CA) diluted 1:400; and stained corneas were stored in 100% glycerol at 4°C. Stained whole corneas were photographed with a digital camera (Coolpix 995; Nikon, Tokyo, Japan) mounted on a dissecting microscope (Wild M5; Martin Microscope Co., Easley, SC) and transmitted light from a light source placed below the glass stage. For staining corneal nerves in sectioned corneas, dissected trimmed fixed corneas were rinsed twice for 20 minutes each in PBS, embedded in paraffin as described,29 and sectioned perpendicular to the anterior–posterior corneal axis at 20 μm. Sections were mounted on slides, rehydrated, rinsed twice for 10 minutes each in PBS, blocked for 50 minutes at room temperature in SalG+0.2% Triton X-100+2% powdered milk (BlkSln), incubated 60 minutes at room temperature in chick nerve-specific antibody CN31 diluted 1:4 in BlkSln, rinsed four times for 15 minutes each in BlkSln, incubated 60 minutes in Cy3-conjugated goat anti-mouse IgG + IgM (H+L; Accurate Chemical & Scientific Corp., Westbury, NY), diluted 1:100 in BlkSln, rinsed three times for 15 minutes each in PBS, mounted (Vectorshield; Vector Laboratories, Burlingame, CA), and viewed with phase-contrast optics and by epifluorescence illumination (Research Standard Microscope; Carl Zeiss Meditec, Inc., Thornwood, NY). Selected fields were photographed (TMAX400 film; Eastman Kodak, Rochester, NY) and developed according to the manufacturer’s specifications. Images were digitized with a scanner (Coolscan 4000; Nikon).

Determining Stromal Anterior–Posterior Nerve Positions in Prestained Whole Corneas

Stained corneas were rinsed twice for 10 minutes in SalG, quick frozen on cryostat chucks in a vertical position in optimal cutting temperature (OCT) compound (VWR; Sakura Finetek, Torrance, CA), and sectioned perpendicular to the anterior–posterior corneal axis at 10 μm with a cryostat (OTF; Hacker-Bright, Fairfield, NJ) at −20°C. Sections were mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA), air dried, covered with a coverslip over 30% glycerol/70% PBS (137 mM NaCl, 2.7 mM KCl, 4.5 mM NaHPO4, 1.4 mM KH2PO4, pH 7.3), and photographed (Coolpix 995 digital camera mounted on a Nikon Diaphot 300 microscope).

Determining Corneal Thickness

Corneas were dissected into SalG, fixed for 20 minutes in 3.7% formaldehyde in SalG, rinsed two times for 10 minutes each in PBS, trimmed, frozen in OCT, and serially sectioned perpendicular to the anterior–posterior axis of the cornea with a cryostat. Ten or more sections across the center of the cornea were viewed (Diaphot 300 microscope), photographed (Coolpix 995 digital camera; Nikon), and assessed with NIH Image software (available at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, NIH, Bethesda, MD).

Quantitating Nerve Growth in Control, T4, 2-TU, and T4+2TU Corneas

All photographs of whole corneas stained with TUJ1 were taken at the same magnification and the same distance from the camera lens. These digital images were made uniform in size and overlaid with a template consisting of five concentric rings of decreasing radius, dividing the cornea into four concentric ring segments and a central region (see Figs. 5D, 7E). The same template was used for all images, so that distances between the concentric rings remained constant relative to one another, and the template was sized so that ring 0 was at the outermost edge of each cornea. For each cornea, the number of nerves that reached each ring was counted.

RNA Isolation and Real-Time PCR

RNA isolation, cDNA synthesis, and real-time PCR were performed as described previously.30 Sequences of genes of interest were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). PCR primers for real-time PCR were designed using software (Designer 31 Molecular Beacons Design; Sigma-Aldrich, St. Louis, MO) to amplify fragments between 80 and 150 base pairs in length, and are listed in Table 1. Gene names conform to guidelines established by the Second International Workshop on Poultry Genome Mapping, 1994 (described at http://www.chicken-genome.org/ provided in the public domain by Roslin Institute, Midlothian, Scotland, UK). Each primer set generates only one amplified band with chick corneal cDNA. For real-time PCR, cDNA dilutions were chosen so that the Ct threshold for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was between 13 and 16. All comparative real-time PCR reaction series consisted of duplicates for 1× and a 1:10 serial dilution of cDNA for each PCR primer pair. Primer pair efficiencies for this 10-fold cDNA dilution were between 90% and 110%. GAPDH expression was chosen for normalization of all gene expressions. Because corneal GAPDH expression was greater than the expressions of any of the other genes included in this study, graphic representation of the normalized data employs a negative y-axis.

Results

Progress of Innervation

Nerves did not penetrate the anterior corneal stroma until E9 and then progressed tangentially toward the epithelium, but never invaded the posterior stroma. Bee6 originally used a modified Bodian metallic copper staining method to visualize periocular and corneal nerves and observed nerves entering the avian cornea on E10. With the more sensitive anti-neuronal β-tubulin-specific TuJ1 antibody and visualization with horseradish peroxidase-conjugated secondary antibody, corneal nerves were seen bending their defasciculations away from the edge of the cornea (Fig. 1: E7, E7a, E7b, E8, E8a; white arrowheads) or actively growing away from the cornea (Fig. 1: E8, E8b; black arrowheads) on E7 and E8, as the main axonal nerve...
trunks extended dorsally and ventrally around the cornea. But then on E9, nerve growth cones found the cornea permissive of defascicular extensions (Fig. 1, E9; black arrows), and no longer bent aside as they entered the corneal matrix (Fig. 1: E9a, E9b; black arrows). On E9 the individual nerve fibers were widely spaced from one another and had not defasciculated extensively (Fig. 1: E9a, E9b; black arrows). E10: the depth of nerve penetration increased, and individual nerves were increasing their defasciculations (E10, E10a, E10b). E12: continued defasciculations increased the nerve density along the radius of the cornea (E12, E12a) as nerves approached the center of the cornea, but did not yet reach it (E12, E12b). E14: the nerve web along the cornea radius was very complex (E14, E14a), and nerves now reached across the center of the cornea (E14, E14b). Scale bar, 0.5 mm.

**Corneal Expression of Growth Factor and Regulator Genes**

Corneal expressions of major growth factor and axon pathfinding regulator genes for TG neurons do not change significantly from E7 to E12 as the cornea changed from nonpermissive to nerve growth cone permissive. To determine whether corneal nerves are suddenly able to invade the cornea on E9 because corneal cells abruptly increase expressions of genes for TG neuron neurotrophins or, conversely suddenly downregulate expressions of TG neuron chemorepellants, RNA isolates from E5, E7, E9, E12, and E18 corneas were examined by real-time PCR for expressions of selected candidate genes. PCR primers are listed in Table 1.

**Corneal expression of the neurotrophin genes NGFB and NTF3** increased 5- to 7.5-fold from E5 to E7, but then did not change from E7 to E12 (Fig. 3A). NTF3 expression remained high through E18, whereas NGFB expression declined slightly from E12 to E18. BDNF was essentially not expressed in the chick cornea. From E9, when TG axons first entered the cornea, through E18, expressions of NGFB receptor NGFR and NTF3 receptor NTRK3 rose 20- and 10-fold, respectively, in contrast to expression of NTRK2, which did not increase until after E12, and expression of NTRK1, which remained low and did not increase appreciably during corneal development (Fig. 3A). Of the corneal epithelium (Figs. 2E, 2F), At no time were nerves seen in the posterior corneal stroma or endothelium.
mon Schwann cell markers, myelin Po (MPZ), a marker for both myelinating and nonmyelinating Schwann cells in chick embryos, expression remained fairly constant until E9, and then rose 10-fold from E9 to E18, whereas genes for other Schwann cell markers, Substance P (now identified as tachykinin, TAC1) and glial fibrillary acidic protein (GFAP), were very minimally expressed in the cornea (Fig. 3B). Neuregulins are both neurotrophins and survival factors for peripheral nervous system Schwann cells. Expression of neuregulin 1 (NRG1) sensory and motor neuron-derived factor isoforms 1a and 2b increased from E9 to
E18 at a rate that closely paralleled the increase in MPZ expression, although 2α was less highly expressed, but rose 5- to 7.5-fold from E9 to E18 (Fig. 3B). Similarly, expression of the neuregulin receptor genes ERBB2 and ERBB4 increased virtually in parallel with the increase in MPZ expression from E9 through E18, and ERBB3 expression, while lower than that of ERBB2 and ERBB3, also rose fivefold from E9 through E18 (Fig. 3B).

Semaphorins and their receptors are chemoregulators for sensory and TG-neuron pathfinding during development.22–24 Chick corneal semaphorin 3A and 3F (SEMA3A and SEMA3F) gene expressions did not change significantly between E7 and E9, when the cornea transitioned from nonpermissive to permissive for nerve penetration (Fig. 4A). SEMA3A expression increased slightly, whereas SEMA3F expression declined slightly from E9 to E12. After E12, expressions of SEMA3A, -3F, and -7A declined significantly by E18. In contrast, expressions of SEMA3E, -4D, and -4G actually increased approximately 10-fold each between E9 and E18. SEMA5A, the most highly expressed corneal semaphorin, was two- and fivefold more highly expressed than SEMA3A and -3F, respectively, at E9; 3- and 10-fold more highly expressed than SEMA3A and -3F, respectively, at E12; and remained the most highly expressed SEMA at E18 (Fig. 4A). Expressions of SEMA3B, -3C, -3D, -6A, -6D, and -7A did not change significantly from E9 to E18 as the nerves defasciculated and grew extensively in the cornea (Fig. 4A). Semaphorin 3 ligands react with the receptors neuropilin 1 and 2, and the plexins.34 Semaphorin 4, -5, and -6 ligands bind plexins alone, and semaphorin 7 ligands bind integrins.34 Neuropilin-1 and -2 (NRP1 and NRP2) genes were expressed at a relatively high constant level from E7 to E12 as TG nerves penetrated and grew into the cornea, then declined from E12 to E18 (Fig. 4B). Similarly, expressions of the plexin receptor genes PLXNA2, -A4, and -B1 remained the same from E9 to E12, then declined from E12 to E18, whereas PLXNA1 (the most highly expressed PLXN in the cornea), PLXNB2, -C1, and -D1 expressions did not change significantly from E9 to E18 (Fig. 4B).

Another family of pathway chemoregulators for TG neurons are Eph tyrosine kinase receptors and their ephrin ligands.5 Chick corneal EPHA3 and -A4 gene expressions increased 10-
and 5-fold, respectively, from E7 through E12 and then expression of EPHA3 plateaued, whereas expression of EPHA4 increased another twofold by E18 (Fig. 4C). Expressions of EPHA1, A9, and B3 were significantly lower than the expressions of EPHA3 and A4, and they remained constant from E9 to E12, but then they also rose approximately 7.5-fold from E12 to E18. In contrast, expressions of EPHA5, A6, A7 (the most highly expressed receptor), A8, B1, B2, and B6 varied widely in their respective levels of expression, but did not change their levels of expression significantly from E9 to E18 (Fig. 4C). Eph receptor ligands EFNA2 and A5 were very highly expressed in the cornea from E7 through E18 and did not change their level of expression during the time the cornea transitioned from nonpermissive to permissive for nerve growth (Fig. 4D). Expressions of EFNBI, B2, and A6 were the same or lower than expressions of EFNA2 and A5, and they also remained unchanged from E9 through E18 (Fig. 4D). Thus, EPH and EFN expressions either increased or stayed the same as the cornea transitioned from nonpermissive to permissive for nerve growth.

Effect of Embryonic Exposure to T4 on the Rate of Innervation

Precocious embryonic exposure to T4 increased the rate of nerve penetration into the cornea, but did not change the circumferential or anterior-only distribution of nerves in the cornea. When precociously administered to the embryo at high levels, T4 increases corneal thinning and transparency.16,17,19 Similarly, precocious administration of T4 on E8 increased the rate of nerve penetration into the cornea by E11, such that nerves covered a greater proportion of the cornea in T4-treated embryos than in control embryos (Fig. 5). T4 stimulation of nerve growth was dose-dependent, with administration of 5 μg T4 causing greater stimulation than administration of 2.5 μg T4 (Figs. 5B, 5C). To quantitate these observations, a concentric ring template was drawn and superimposed on the photograph of each stained cornea (Fig. 5D), then the number of nerves reaching each concentric ring was counted and presented graphically (Fig. 5E). Whereas almost no nerves penetrated as far as ring 3 in control corneas, a few nerves reached ring 3 in corneas treated with 2.5 μg T4, and many nerves reached ring 3 in corneas from embryos treated with 5 μg T4. In addition, nerves defasciculated more extensively in the presence of increased T4 than in control conditions. Thus, there was a greater number of nerve fibers reaching rings 1 and 2 in corneas from embryos treated with 2.5 μg T4 than in the control corneas and more still at each of these penetration distances in corneas from embryos treated with 5 μg T4, although there was an overlap in the standard deviation bars for the number of nerves reaching ring...
corneas revealed that precocious T4 treatment also did not reduce corneal nerve defasciculation. In contrast, T4 administered alone stimulated nerves to extend over approximately one-half to two-thirds of the corneal surface (Fig. 7C). Nerve defasciculation in the peripheral cornea was significantly increased compared both to control and 2-TU-treated corneas, at both rings 1 and 2 (Fig. 7F; compare Figs. 7Aa, 7Bb, 7Cc). Surprisingly, however, when administered together on E8, T4 + 2-TU stimulation of nerve penetration and defasciculation was additive, such that together they stimulated nerves to extend over two-thirds to four-fifths of the cornea, with some nerves extending all the way to and across the center of the cornea by E11 (Figs. 7D, 7Dd; arrowheads), and significantly more nerves reached rings 1, 2, and 3 than in T4-treated, 2-TU-treated, or control corneas (Fig. 7F). In cross-sectional views, it can be seen that nerves in corneas stimulated with T4 + 2-TU were still restricted to the anterior corneal stroma and did not invade the posterior stroma (Fig. 6C; arrows), as was the case for normal corneal innervation and for innervation stimulated by T4 alone. Failure of 2-TU alone to prevent nerve penetration into the cornea and 2-TU augmentation of T4-stimulated corneal nerve growth contrasted with 2-TU’s ability, in the presence of T4, to prevent the compaction that exposure to T4 alone causes in corneas (Fig. 8). Indeed, in many cases T4 + 2-TU caused corneas to become even thicker than control corneas (Fig. 8), as T2U does in corneas from embryos exposed to 2-TU alone. This demonstrates that in embryos treated with T4 + 2-TU, 2-TU functions in its expected capacity as an inhibitor of corneal thinning, even as it is acting as a stimulator of nerve growth. These results suggest that T4 stimulates nerve growth in the cornea by a different mechanism than it uses to stimulate corneal compaction.

In conjunction with stimulating corneal thinning and increased transparency, T4 stimulates increased corneal expressions of mRNAs for thyroxine hormone receptor β and carbonic anhydrase II (CA2) and decreased corneal expression for galactose sulfate transferase 1 mRNA. In contrast, E8 treatment of embryos with T4 did not alter corneal expressions of genes for the neurotrophins that stimulate NC-derived TG axon growth, NGFB, NTF3, or BDNF, or of their receptors, TRK1, -2, or -3 or NGFR, of the Schwann cell marker genes MPZ or GFAP, or of the Schwann cell neurotrophin gene NRG1 isoforms 1a or 2b (Fig. 9). Indeed, only expression of the Schwann cell marker TAC1 was changed, and it was reduced approximately eight-fold. However, corneal expression of TAC1 was extremely low in the cornea under normal conditions (Fig. 3B), and so this reduced expression is unlikely to influence Schwann cell function in the cornea significantly.

These observations suggest that the T4 nerve-stimulating mechanism does not involve alteration of the expressions of nerve growth stimulating genes that were already highly expressed in the cornea.

T4 itself binds integrin αV/β3 receptors more avidly than these receptors are bound by T3 and stimulates MAPK-signaling pathways. JTGAV was expressed in the cornea approximately 20-fold more than ITGB3 (Fig. 10). Their respective expression levels rose in parallel from E5 to E7 and then did not change appreciably from E7 to E12. After E12, expression of JTGAV increased approximately two-fold, whereas expression of ITGB3 decreased approximately seven-fold.

**Discussion**

NC-derived TG nerve axon fascicles reached the ventrotemporal edge of the embryonic chick cornea by E6, but did not enter the cornea until E9. Then suddenly, on E9, nerves entered the
anterior corneal stroma from all sides of the cornea at once, and extended in tangential directions toward both the center of the cornea and the epithelial layer on the anterior surface of the cornea. Nerves never entered the posterior corneal stroma or innervated the endothelium. What changes so dramatically on E9 that converts the cornea from non-neuronal growth cone-permissive to nerve growth cone-permissive?

Changes in nerve-related gene expressions in the cornea do not support their putative role in causing the E7 to E9 transition from non-neuronal growth cone-permissive to nerve growth cone-permissive. Corneal expressions of NGB and NTF3 increased from E5 to E7, but did not change significantly after E7. Although Schwann cells can help the pathfinding of nerve axons,27 expression of the Schwann cell marker gene MPZ actually decreased from E7 to E9, before beginning its increase from E9 through E18. Our data showing increasing corneal expressions of the neurotrophin receptor genes TRK1, -2, and -3 and NGFR during development suggest that mRNAs for all these genes exist in TG afferent axons and/or growth cones as they invade and populate the cornea. mRNAs for some cytoskeletal proteins36 and signaling molecules, such as RhoA,36 are localized in axons and growth cones, and growth cone translation of RhoA is necessary for SEMA3A-mediated growth cone collapse.38 mRNAs for NGFR and TRKs have been identified in growth cones of embryonic rat hippocampal neurons in culture.59 In cultured E8 chick forebrain neurites, NTF3 stimulates β-actin mRNA localization into growth cones, suggesting that neurotrophins can regulate the axonal transport of mRNAs in embryonic neurites.40 NTF3 is the most highly expressed neurotrophin gene in the cornea, so NTF3 may stimulate transport of NGFR and TRK mRNAs into TG afferent growth cones. There is, as yet, no evidence of NRG1 mRNA expressions in nerve growth cones,41 but NRG1s may also be synthesized by Schwann cells themselves and function in an autocrine or paracrine manner, to induce Schwann cell proliferation.42 Various NRG1 splice variant mRNAs and their receptor ERBB2, -R3, and -B4 mRNAs have been identified in adult human corneas.43–45 Type IV collagen- and substance P-immunopositive Schwann cells have been identified in the chick cornea from E10 onward.46 Our observations that corneal NRG1s and MPZ expressions increased in the cornea from E9 supports the idea that Schwann cells accompany TG afferents into the corneal stroma from their earliest corneal penetration, but does not resolve whether Schwann cells precede or follow TG afferents into the cornea.

Like developing tooth primordia,47 the developing cornea expresses a wide array of semaphorins at various levels and in various temporal patterns. Semaphorins have significant pathfinding effects on NC cells and axons: SEMA3A on sympathetic nerve pathfinding46,49 and corneal nerves,24 SEMA3E in capil-

FIGURE 7. Precociously administered 2-TU did not block corneal nerve penetration and produced additive stimulation when administered with T4. SalG, 2-TU, T4, or T4+2-TU were administered in ovo on E8; corneas were dissected on E11, fixed, and stained with anti-neuronal β-tubulin-specific TuJ1 antibody. (A, Aa) Cornea from SalG-treated embryo: nerves covered approximately one-fourth to one-third of the corneal surface, with few defasciculations and much of the center of the cornea not penetrated by nerves (Aa). (B, Bb) Cornea from 2-TU-treated embryo: nerves covered approximately one-fourth to one-third of the corneal surface; (Bb) showed very little defasciculation, and still left much of the center of the cornea unpenetrated. (C, Cc) Cornea from T4-treated embryo: nerves covered about one-half to two-thirds of the corneal surface, (Cc) were more defasciculated around the cornea, and left less of the center of the cornea unoccupied. (D, Dd) Cornea from T4+2-TU-treated embryo: nerves covered approximately two-thirds to four-fifths of the corneal surface and (Dd) some crossed the center of the cornea. (E) A concentric ring overlay was superimposed on each cornea, and the number of nerves reaching each ring was counted. (F) Summary graph of the number of nerves that reach each penetration ring in control, 2-TU, T4, and T4+2-TU-treated corneas. Control and T4: n = 8; 2-TU: n = 6; (T4+2TU): n = 9. Scale bar, 0.5 mm.
lary endothelial tip cell pathfinding. SEMA3F in facilitating TG formation in the head. SEMA4D in hippocampal neuron remodeling. SEMA5A in diencephalon axon pathfinding. SEMA6C and SEMA6D in dorsal root ganglia and PC12 neurite extension. SEMA7A in central and peripheral axon pathfinding. Semaphorin guidance is usually repulsive, but can become attractant in the presence of high cyclic nucleotide concentrations, and can even be repellant to axons, yet attractant to dendrites in the same cortical neurons. How-

ever, corneal expressions of SEMA3A and -3F remained high between E7 and E12, before showing significant decreases in expression, and other soluble and membrane-bound SEMA corneal expressions were essentially unchanged through corneal development, declined only after E12, and some did not decline at all. Moreover, it is unlikely that corneal TG axons switched from a repellant to an attractant response to SEMA3A during the E7 to E9 corneal transition period, as adult rabbit TG afferents retain their repulsive response to semaphorin III. Individual SEMAs react with specific NRP or PLXN receptors, such that a specific SEMA can change from a neurorepellant to a neuroattractant, depending on which specific NRP or PLXN a neuron expresses. However, in developing corneas both NRP1 and NRP2 and most PLXN expressions remained constant relative to each other from E7 through E12, before declining markedly, so a significant change in TG afferent receptor expression seemed unlikely to be the reason why TG afferents could suddenly invade the cornea at E9 when they could not at E7. Notably, SEMA3E, -4D, and -4G expressions rose during corneal development, while NRP and PLXN expressions were falling. Both SEMA3E and -4D stimulate PC12 neurite outgrowth in the presence of NGFB, using MAPK signaling. SEMA4G possesses an RGD integrin-binding do-

main like the one used by SEMA7A to bind integrin, activate an MAPK pathway, and stimulate central and peripheral axon pathfinding. It is therefore possible that TG afferent growth

Figure 8. 2-TU inhibited corneal compaction while stimulating corneal nerve penetration. SalG, T4, or T4+2-TU were administered in ovo on E8; corneas were dissected on E11, fixed, trimmed, frozen in OCT compound, and serial sectioned on a cryostat. Sections were mounted on slides, viewed with phase-contrast optics, and photographed. Stromal thicknesses were measured from digital images of the sections. Each data point represents an individual cornea. T4 caused increased corneal thinning, compared with SalG controls, but 2-TU prevented T4-stimulated corneal thinning, and sometimes caused corneal swelling compared with SalG controls. Control: n = 4; T4: n = 5; (T4+2TU): n = 6. Error bars, SD determined from the range of measurements made from successive serial sections across a single cornea.

Figure 9. T4 stimulation of corneal nerve growth was not accompanied by increased expressions of neurotrophin, neurotrophin receptor, Schwann cell marker, or Schwann cell neurotrophic genes in the cornea. Corneas from embryos exposed precociously to SalG (Controls) or 5 μg T4 for 3 days were dissected, trimmed, and extracted for whole-cell RNA. cDNAs were synthesized from each pool, and the relative abundances of mRNAs of genes of interest were determined with real-time PCR. Results were normalized to GAPDH expression. Control expressions were subtracted from T4 expressions. Gene expressions assessed: NGFB, NTF3, BDNF, TRK1, TRK2, TRK3, NGFR, MPZ, TAC1, GFAP, NRG1-1a, and NRG1-2b. Gene names are listed in Table 1. Error bars, SD.
in E12 to E18 chick corneas is stimulated by SEMa3E, -4D, and -4G, using a MAPK signaling pathway accessed by receptors other than NRPs or PLXNs.

The absence of ENFA2/A5 expression in periocular mesenchyme permits EPHA3-expressing TG afferents to extend into the periocular mesenchyme from E3 through E8. Yet corneal expressions of EPHA3 and -A4 increased from E7 to E12, ENFA2 and -A5 expressions remained high from E7 through E18, ENFB2 was equally highly expressed through E18, and expressions of EPHA1 and -A9 began low and increased significantly as the cornea developed. Therefore, one must ask why EPH-expressing TG afferents suddenly became tolerant to/stimulated by ENFs in the cornea. In fact, such bifunctional ENFB cueing governs avian NC cell migration in the HH14 and HH23 trunk. These observations suggest that EPH downstream signaling determines the eventual outcome of EPH-ENF guidance, and ultimately depends on receptor cell expression of such things as specific EPH isoforms, Rho, Rac, Cdc42, or RAS/MAPK signaling intermediates. Intriguingly, neurons can change from repellant to tolerant EPH-ENF regulation if their EPHs cease to be embedded in their growth cone membranes, but instead are embedded only on their axonal membranes. It remains to be determined which mechanisms are active in the cornea, but the fact that both ENFA4 and ENFB isoform expressions remained high throughout corneal development suggests that EPH-ENF guidance plays a positive role in corneal nerve growth.

When nerves enter the cornea prematurely, they enter predominately from the ventrotemporal side. Since precocious T4 stimulation caused neither one-sided corneal nerve penetration nor loss of anterior-only penetration, we concluded that T4 stimulation did not lead to premature corneal nerve penetration. T4 nerve stimulation was, however, not inhibited by 2-TU, even though 2-TU inhibited corneal thinning. Indeed, adding 2-TU simultaneously with T4 augmented the extent of nerve penetration into the cornea, compared to either T4 alone or 2-TU alone. This suggests that T4 stimulation of corneal nerve growth may use T4-dependent mechanisms different from those used in T4 regulation of corneal thinning. In vivo, T4 is converted to its nuclear thyroid hormone–activating form, T3, by the deiodinases DIO1 or -2, and to biologically inactive rT3 by DIO1 directly, and inhibits iodination of precursors to form T3 in vivo. Thus, in the presence of 2-TU, T4 is not converted to T3 or degraded to rT3 as readily in the cornea, and its effective concentration becomes higher. In addition, antithyroid drugs like 2-TU also inhibit hormone receptor-mediated transcription, so the T4-induced increase in Dio3 transcription probably does not occur in the presence of 2-TU, further increasing effective T4 concentration in the presence of 2-TU. Moreover, T4 stimulates DIO2 degradation, again decreasing the conversion of T4 to T3 and further contributing to an increase in T4 concentration in tissues treated with 2-TU and T4 simultaneously. Therefore, since T4 stimulation of corneal nerve penetration was concentration dependent (see Fig. 5), nerve penetration was stimulated to a greater extent in embryos treated with T4 + 2-TU than in embryos treated with T4 alone. In contrast, observations that both 2-TU alone and T4 + 2-TU inhibited corneal thinning suggest that T4 conversion to T3 and subsequent T3-thyroid hormone receptor–mediated transcription regulation are necessary for corneal thinning, a conclusion consistent with our previous observation that CA4 mRNA transcription is greatly increased in corneas isolated from embryos precociously treated with T4. The reason 2-TU alone does not increase corneal nerve penetration from E8 to E11 is probably due to the fact that there is very little T4 in the developing chick eye during this interval.

An alternative mechanism for T4 action in nerve growth stimulation could derive from observations that T4 increases actin polymerization in the brain and in cultured astrocytes, in which actin cytoskeleton polymerization has been shown to be necessary for neurite outgrowth. In addition, T4 binds ITGAV/ITGB3 receptors on cell surfaces in a manner that activates the MAPK pathway. In cultured glioma cell lines, T4 acts as a growth factor using this pathway. Our results show that T4 stimulation of corneal nerve growth did not change expressions of neurotrophins in the cornea and that cells in the cornea expressed ITGAV and ITGB3, although we cannot yet say which cells in the cornea express these integrins. It is consistent with our data, however, to propose that T4 stimulates the rate of nerve growth in the cornea by way of a nerve membrane ITGAV/ITGB3/MAPK-signaling pathway that results in increased actin cytoskeleton polymerization in TG afferent axons. Indeed, as NRP and PLXN expressions decline in the cornea, nerve membrane ITGAV/ITGB3 or may also become more available to regulate SEMa3B, -4D, and -4G guidance of TG- afferents positively, once they enter the cornea.

T4 clearly increased nerve elongation in the cornea when present in sufficient concentrations. However, it is unlikely that T4 is involved in normal embryonic chick E9 corneal transition from nerve growth cone nonpermissive to growth cone permissive, because in the developing chick embryo T4 concentrations from E8 to E9 are undetectable, the embryonic...
chick thyroid gland does not begin to produce T4 until E9, and T4 is not detectable in the eye until E10.14,15 Even when T4 concentration was precociously raised in embryos at E8, nerves did not enter the cornea asymmetrically, as they do when normal embryonic corneal nerve growth cone nonpermissive signals are precipitously interrupted.24 Corneal expressions of major neurotrophins and pathfindig molecule genes also did not change significantly during this transition period. But what does change markedly at this time is the corneal rate of synthesis and ECM deposition of highly sulfated KSPGs.11,12 Before E9, highly sulfated KSPG is located only within corneal stromal keratocytes, but on E9 it suddenly appears extracellularly in the posterior stroma and then accumulates extracellularly as a wave across the stroma, such that by E11 the posterior half of the stroma, by E13 the posterior three-fourths of the stroma, and by E16 the entire stroma stains densely for extraacellular highly sulfated KSPG. In addition, corneal concentrations of monosulfated plus disulfated KS disaccharides are lowest at E10 and rise to maximum concentrations by E14.75 Lwigale and Bonner-Fraser23 have recently suggested that it is lens-produced soluble SEMA3A diffusing across the cornea that repels TG nerves during early development and have shown that, without the lens, nerves penetrate the cornea before E9. They conclude that nerves penetrate the cornea when "something" makes a barrier to the entry and diffusion of lens-produced SEMA3A across the cornea. The great increase in synthesis and deposition of highly sulfated KSPG into the ECM in the posterior stroma could provide such a barrier, because it starts to occur on E9, just when nerves began to enter the anterior corneal stroma. KSPGs are very large, highly negatively charged molecules that can act as ion exchange resins, bind molecules such as SEMAs,74 and even change the functionality of some of the molecules they bind. Indeed, the functionality of SEMA5A, product of the most highly expressed SEMA in the cornea, changes depending on which ECM proteoglycan it binds. SEMA5A is an axon chemoattractant when bound to KSPG, which could also simultaneously avoid areas where highly sulfated KSPGs are localized.80 So, when diffusion of lens-produced SEMA3A becomes blocked by ECM KSPG in the posterior cornea, NC-derived TG axon growth cones may enter the anterior stroma preferentially, to keep away from the highly sulfated ECM KSPG accumulating in the posterior stroma, and then subsequently may extend tangentially toward the epithelium to continue avoiding the advancing front of highly sulfated ECM KSPG, as it accumulates across the cornea. As the stromal ECM becomes heavily occupied by highly sulfated KSPG, which could also simultaneously be binding and converting highly expressed membrane-bound SEMA5A from chemoattractant to chemorepellent, corneal nerves extend from the anterior stroma into the epithelium and spread out in the epithelium. Thus, ECM KSPG could determine which nerve growth regulating molecules are able to enter the cornea from other tissues and the molecular configurations of nerve growth–regulating molecules throughout the cornea, as well as which areas of the cornea become suitable for nerve growth cone attachment and movement. In this way, KSPG could determine both when and where nerves grow in the cornea.

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