Specificity of corneal nerve positions during embryogenesis

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Purpose: Embryonic corneal innervation first involves pericorneal nerve ring formation, with nerves in specific positions, followed by innervation of the corneal stroma from the ring. Here we determine whether nerve bundles enter the cornea at specific locations along the ring and whether bundles enter the cornea at specific depths.

Methods: Whole mount embryonic quail corneas immunostained for nerves were scanned using confocal laser microscopy. Images were superimposed digitally in pseudo-colored pairs to detect similar positions of innervation, and then rotated stepwise to determine if degree of synchrony was decreased. Degrees of innervation of each corneal quadrant were quantified. Depths of stromal bundles innervating the cornea were determined by depth of focus analysis.

Results: Superimposition of images indicated many nerve entry points in similar locations, suggesting specificity. However, stepwise rotations of one image above the other revealed that degree of positional synchrony remained constant, suggesting that nerves do not occur in specific locations, but rather simply at approximately equal distances around the cornea. Corneas from both left and right sides are innervated by similar numbers of nerve bundles (Left, 44±0.4; Right, 44±1.0), with the same number/quadrant (Left, 11±0.2; Right, 11±0.2). Nerves entering the stroma closest to Descemet’s layer innervate either the entire cornea along that radius or only the central-peripheral and central cornea; those entering nearer Bowman’s layer innervate only peripheral cornea.

Conclusions: Avian corneal nerve bundles enter along radii spaced at equal intervals along the pericorneal nerve ring, suggesting an innervation mechanism based on equal spacing between nerves. Nerve bundles from the nerve ring enter the stroma at depths correlated with their subsequent targets.

Sensory nerves originating from the trigeminal ganglion extensively innervate the entire surface of the cornea, making it one of the most highly innervated peripheral tissues of vertebrates. Several studies suggest that corneal nerves play major roles in maintaining the integrity of the corneal epithelium and transparency in adults [1-5], but apparently are not required for corneas to become transparent during avian embryogenesis [6]. Dependence of corneal functions on innervation has been summarized recently [7].

During embryonic development of the chick, nerves from the neural crest component of the trigeminal ganglion [6] send growth cones along the ophthalmic branch of the ganglion to first make a complete ring around the cornea [8], in a manner that generates nerves in specific positions within the ring [9]. At a sharply defined time in development, nerves then emanate from many points along the pericorneal nerve ring and radially enter the corneal stroma. In humans, the nerve bundles enter the middle stroma [10,11], but enter deeper layers of the stroma in the eyes of other animals [12]. In both groups, the corneal epithelium eventually contains extensive nerve terminals, but neither the posterior stroma nor the endothelial cell layer receives such innervation. Stromal nerves consist of large bundles (fascicles) of mostly nonmyelinated [7] axons that extend into the center of the cornea and smaller bundles that innervate only the periphery of the cornea. As the large nerve bundles extend toward the center, they branch into smaller bundles that rise toward the epithelium, first traversing Bowman’s layer and the basement membrane. Smaller stromal nerves turn sharply toward the surface upon entering the cornea, penetrate the epithelial basement membrane and form basal epithelial lashes [13] which divide into finer beaded and straight axons that terminate among the basal and wing cells of the epithelial layer [14-16].

The anatomy and morphology of corneal innervation have been studied extensively in several animals (reviewed by Marfurt [7]). Briefly, avian corneas have been used to demonstrate the embryonic pattern of corneal innervation [8,9], as have embryonic human corneas [17], whereas mammalian (mouse, rat, cat, rabbit, dog, monkey, and human) corneas of adults have been used to determine neural organization [10,18] and its remodeling [19], differences in neurochemical content [12,15,20-22], and effects of denervation on corneal function [1,7,23,24]. To our knowledge, the positional specificity of corneal nerve entry points into the stroma and depths of innervation have not been investigated previously.

In this study we use fluorescent immunostaining together with confocal microscopy and computer imaging programs to analyze the positional specificity of corneal nerve entry points and distribution throughout its thickness, as well as quantify corneal innervation in different regions of the late embryonic avian cornea. Our data suggest that there are no specific nerve entry points, but that the nerves are very evenly distributed around the entire corneal circumference. In contrast, there is specificity within the corneal thickness, such that stromal nerves innervate the entire cornea, with short stromal nerves branching and rising steeply to innervate only the periphery,
and long nerves running almost parallel to Descemet’s layer, either innervating the entire corneal radius or only the central- peripheral and central parts.

**METHODS**

*Whole mount immunostaining:* Corneal nerves were immunostained according to the method of Koo, et al. [25]. Briefly, embryonic Day 16 (E16) Japanese quail (Coturnix japonica) embryos (one day before hatching), were euthanized by severing the spinal cord at the base of the head, in accord with the National Research Council recommendations and in accord with a protocol approved by the Kansas State University Animal Care and Use Committee; intact eyeballs were removed, rinsed in phosphate buffered saline (PBS), and fixed overnight in 4% paraformaldehyde in PBS at 4 °C with tumbler. Anterior halves of eyes were dissected and trimmed, rinsed in PBS, then stained for nerves using either a quail specific monoclonal antibody, QN (mouse IgG1) [26], at 1:1 dilution or mouse monoclonal anti-neuron-specific β-tubulin antibody TuJ1 (IgG2a, BABC0, Richmond, California) in antibody buffer (PBS containing 0.2% [w/v] bovine serum albumin, 0.2% [v/v] Triton X-100, and 5% [v/v] heat inactivated goat serum). Corneas then were incubated overnight at 4 °C in secondary antibody (Alexa Fluor 488 anti-mouse IgG1 for QN and Alexa Fluor 488 anti-mouse Fab2 fragments for TuJ1, Molecular Probes, Eugene, Oregon) used at 1:200 dilution in antibody buffer. Stained corneas were rinsed in PBS, then mounted on slides using 50% (v/v) glycerol in PBS. Corneal nerve images were captured using a laser confocal microscope (Zeiss LSM 410, Oberkochem, Germany).

*Image analysis:* Six different embryos were used and, from these, corneal nerve images from six right and six left embryonic corneas were captured using confocal microscopy and imported into Adobe Photoshop 5.5 (Adobe Systems, Inc., Mountain View, CA), for manipulation. Images of nerves were highlighted using the “Magic Wand” feature of Adobe Photoshop. Images of the corneal nerve bundles were made distinct from the other tissues of the cornea by changing the pseudo-colors of the nerves and graying the remaining portion of the corneas so the entry locations of the nerve bundles appeared more distinct. Each corneal nerve image then was subjected to four tests.

*Superimposition test:* Corneal nerve images, from the same side of two different embryos, and as viewed from the endothelium side of the cornea, were superimposed digitally to determine the degree to which positions of corneal nerve bundle ingrowth occurred at the same locations in the two corneas. The overlaid corneal images were lined up in register by overlaying the chorioid fissure site of one with that of the other. Nerves that superimposed exactly on top of one another therefore would be judged to be in the same fixed position. Such synchrony of nerve bundle innervation site locations was easily revealed by making the nerves of one cornea one pseudo-color (e.g., red) and those of the other cornea a different pseudo-color (e.g., green), such that regions where the images of the two sets of nerves overlapped exactly were in the same position, and their combined image therefore became a third pseudo-color (e.g., yellow, a “color-changed” image). The positions of any nerve bundles in the combined image that were thereby transformed to a third pseudo-color were then recorded, first, for the “original, non-rotated” image pairs or sets. Each corneal nerve image was thereby compared, one on one, by direct overlay superimposition with each of the five other independent images of corneas from that same side (left or right). This generated 15 pairs of compared images from each side.

*Rotation test:* In each of the above pairs of superimposed images, one component image then was rotated with respect to the other member of that pair by successive steps of 90° to determine if the degree of exact nerve superimposition between the two images changed significantly. If the nerve bundle innervation sites determined by the superimposition test are in the same specific location in the two corneas (color-changed nerves in the combined, superimposed images), then rotating the image of one of the corneas with respect to the other should eliminate or significantly reduce the degree to which such nerve bundle innervation sites are seen to overlap. The number and location of such color-changed nerve bundles after such image rotations were recorded for each 90° rotation interval for comparison against each other and against the data from the original, non-rotated pairs.

*Reflection test:* For the reflection test, six additional corneas (from 3 embryos) were marked so that right and left corneas from the same embryo could be identified. Corneal nerve images of right corneas were superimposed on those of left corneas from the same embryo. The patterns should be mirror images rather than duplicates of one another and therefore should reveal no color-changed nerves. In addition, one image of each such pair was electronically reflected (“flipped”) about its dorsal-ventral axis and used again in superimposition. Stepwise rotation of one image with respect to the other, as in the rotation test, should decrease the number of color-changed nerves after one image has been flipped.

*Quadrant test:* In addition, to quantify innervation by corneal region, each corneal image was divided into four distinct quadrants, which were oriented with respect to the choroid fissure. Confocal microscopy was performed at the optimal depth to view both long and short nerves that innervate the cornea. The numbers and locations of ingrowing nerve bundles then were recorded in each quadrant in six left and six right corneas, allowing quantification of innervation around the entire cornea.

*Statistical analysis:* The Mann-Whitney U test was performed in the superimposition, rotation, reflection, and quadrant tests to detect a statistical difference (α=0.05) in the numbers of nerve trunks entering corneas from the pericorneal nerve ring.

**RESULTS**

*Direct overlay of pairs of images:* Overlaying pairs of corneal nerve images showed a significant number of color-changed nerve trunks (indicating nerves in identical positions in superimposed images), as well as several pairs in very close, although not identical, positions (Figure 1A-C). Such syn-
chrony or near synchrony of innervation site positions could suggest that ingrowth of corneal nerve bundles occurs at specific locations along the pericorneal nerve ring in each cornea, a reasonable expectation, considering that nerves in the pericorneal nerve ring are in specific locations themselves [9]. However, such positional synchrony, alternatively, might arise simply if innervation occurred at many locations, but at random sites that may or may not be spaced regularly with respect to one another. To distinguish between these two possible mechanisms, one of the corneal nerve images in each superimposed pair was rotated while the second image was maintained in the original position.

**Successive rotation of overlaid images:** The corneal nerve images were subjected to stepwise 90° rotation tests to determine if the synchrony of corneal nerve bundle ingrowth sites arose from a specific pattern of nerve bundle positions, or simply as a result of dense, but random, sites of innervation. Such rotations revealed that the rotated images showed the same degree of nerve bundle overlap (i.e., color-changed nerves) as the original, choroid fissure aligned, images (Figure 1C-F). Thus, in all rotated, superimposed images, there were as many color-changed nerves as in the original, non-rotated, superimposed images. This result therefore negates the original hypothesis that corneal innervation occurs at specific positions. However, there appeared to be approximately equal spacing between nerve bundles growing into the cornea, a geometric arrangement that accounts for why image rotations revealed approximately as many nerve image overlaps as in the original, non-rotated pairs of images.

Nerve images from right and left corneas also were subjected to direct overlay and stepwise rotations, as above. These were therefore comparisons of mirror image patterns, which therefore should have revealed no color-changed nerve matches if innervation occurs in a specific pattern. However, the combined images revealed approximately the same degree of nerve positional matching as observed with corneas from the same side (Figure 2A-C). These direct overlay techniques suggested that in corneas of the same embryo, corneal nerve entry sites were not in specific locations, but rather simply occurred at approximately equal distances from one another around the entire circumference of the cornea.

To convert left and right corneal mirror images to forms that should match one another (if positional specificity exists), one corneal image was flipped electronically around the dorsal-ventral axis, and innervation patterns were once again examined by overlay and rotation of images (Figure 2D-F). Again, the data suggest that corneal nerve images from right and left corneas from the same embryo (one of which is flipped around the dorsal-ventral axis) show approximately the same number of color-changed nerves as those not flipped, suggesting that innervation sites occur at approximately equally spaced distances around the entire cornea, but not in positionally specific sites.

**Quantification of corneal innervation, by quadrants:** The impression of uniformity of innervation around the cornea was reinforced by quantifying the number of nerve bundles innervating each of the four distinct quadrants (Figure 3). Numbers of nerve bundles innervating each quadrant of corneas from both right and left sides were recorded and compared statistically. The data indicate that corneas on each side are innervated by the same number of nerve bundles (Left: 44±0.4,
n=6; Right: 44±1.0, n=6), with the same number of nerve bundles innervating each quadrant (Left: 11±0.2; Right: 11±0.2). In addition, the number of nerve bundles innervating each quadrant of left and right corneas was the same (Table 1 and Table 2). These findings support the hypothesis that the locations of corneal nerve bundle ingrowth from the pericorneal nerve ring are not specific, but rather simply occur at very regular spacing with respect to one another around the entire circumference of the cornea.

Specificity of nerve positions according to distance from the corneal epithelial surface: In the course of using confocal microscopy to record the corneal nerve ingrowth positions described above, it became obvious that nerve bundles enter the cornea at different depths (distances from the epithelium), depending on the subsequent length of the nerve toward the corneal center. Thus, those nerves entering at the deepest levels, farthest from the epithelium and nearest to Descemet’s layer, remained as bundles that traveled farther toward the corneal center than any other nerves. Such nerves, at various locations along a radius, formed branches that turned sharply toward the epithelium and entered that layer, where they branched into fine termini. Conversely, those nerves that entered the cornea at a stromal depth nearest the epithelium were those that grew into the cornea for only a short distance before turning sharply to innervate the epithelium and engage in branching.

To examine the proximal-distal aspect of corneal nerve positions (i.e., distance from the corneal epithelial surface), whole mount E16 quail corneas were immunostained and analyzed by confocal microscopy. Whole corneas (n=8) were initially scanned en face at an optimum focal depth to reveal both long and short nerves. All corneas showed long nerve bundles that either branched shortly after entering the cornea from the limbus (white arrowhead) or approximately halfway between the periphery and center of the cornea (central-peripheral, white arrow), as well as shorter nerves that branched immediately after entering the cornea (Figure 4A, red arrowhead).

A smaller section of the cornea was magnified and scanned from the endothelial side at different focal depths beginning with the basal surface of the endothelium or Descemet’s layer (0.0 µm), and then at different focal depths toward the outer epithelial surface (106.2 µm) of the cornea (Figure 4B-G). The quail corneas at E16 were, on average, about 116 µm thick. Beginning at the basal surface of the endothelium (Figure 4B), the cornea was z-sectioned until the first nerves were in focus (Figure 4C) at about 36.85 µm from the endothelial basal surface. At this focal depth, the nerve ring, long nerves, as well as part of the short nerves (where they branch from the nerve ring) were in focus. The long nerves remained in focus throughout the plane of view, suggesting that they ran almost parallel to the endothelium.

In contrast, the short nerves were in focus only at the branching point from the nerve ring, indicating that they rose to the epithelial surface as soon as they exited the nerve ring. Both long and short nerves were in optimum focus at a focal depth of 116.2 µm from the epithelial surface (106.2 µm), and then at different focal depths toward the outer epithelial surface (106.2 µm) of the cornea (Figure 4B-G). The quail corneas at E16 were, on average, about 116 µm thick. Beginning at the basal surface of the endothelium (Figure 4B), the cornea was z-sectioned until the first nerves were in focus (Figure 4C) at about 36.85 µm from the endothelial basal surface. At this focal depth, the nerve ring, long nerves, as well as part of the short nerves (where they branch from the nerve ring) were in focus. The long nerves remained in focus throughout the plane of view, suggesting that they ran almost parallel to the endothelium.

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depth of about 50.4 μm (Figure 4D), bifurcated into smaller nerve branches at about 65.9 μm (Figure 4E), which traversed through Bowman’s layer and the basement membrane, and then formed ramifications (leashes, Figure 4F) below the basal epithelial layer at about 88.05 μm. The leashes ramified into fine nerve termini (Figure 4G), which innervated the corneal surface.

A schematic cross section of the cornea (Figure 4H) summarizes the observations above, indicating that the avian cornea is innervated by both long and short stromal nerves, which in the limbus region both exit from the nerve ring at the same focal level. The long nerves (blue and black) enter the corneal stroma near its proximal side, closer to the endothelium, and then run in the stroma almost parallel to Descemet’s layer and the endothelium. Some of these bundles of long nerves (blue) begin to branch soon after entering the cornea and innervate the entire corneal surface from the periphery to the center, whereas other long nerves (black) branch later to innervate only the central-peripheral and central surface of the cornea. In contrast, the short nerves (red) enter the stroma at a depth nearer the epithelium, branch immediately, and then rise toward the corneal surface at a much steeper angle to the stroma.

Figure 3. Quadrant definitions of left and right corneas. E16 Japanese quail corneas from left and right sides were immunostained for nerves as in Figure 1, with images captured via confocal microscopy (from endothelial side), digitized, and imported into Adobe Photoshop 5.5. Each cornea was divided into four equal quadrants, using the ventral pole marker (choroid fissure) as a reference point. Each nerve entrance point in each quadrant was counted, allowing quantitative comparison of innervation of each quadrant of left and right corneas (Table 1 and Table 2). Abbreviations: D, dorsal; N, nasal; T, temporal; V, ventral. Bar represents 500 μm.

The number of nerves innervating corneas in Figure 3 is lower than the number in the tables because the image only reveals nerves at one focal depth; the tables include nerve bundles seen at all focal depths. There was no significant difference (a=0.05) in respective pairwise comparisons between quadrants of the same side, between total nerve numbers, or between corresponding left and right quadrants. Abbreviations: CCW, Counter-clockwise; V, Ventral; T, Temporal; D, Dorsal; N, Nasal.

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than the long nerves. They bifurcate and innervate only the peripheral zone of the corneal epithelium.

**DISCUSSION**

This study indicates that nerve bundles from the pericorneal nerve ring do not enter the cornea at specific radial locations around its margin. Rather, they enter as bundles at uniformly spaced sites around the entire circumference of the cornea and at uniform spacing from one another within any quadrant. The mechanisms that account for the timing or for establishing such spacing of innervation remain unknown. Moreover, the depth at which nerves enter the corneal stroma is correlated with the area of the cornea that they subsequently innervate, with the deepest stromal nerves innervating almost the entire corneal surface, whereas the shortest nerves, which enter the stroma nearest the epithelium, innervate only the periphery.

To our knowledge, no studies have been performed previously to determine if nerves enter the cornea only at specific points around its circular margin. Hypothesizing the existence of such specificity in the present study was an extrapolation of the very reproducible positions of nerve bundles seen within the pericorneal nerve ring immediately before it initiates innervation of the corneal stroma at many sites simultaneously [9]. Here, digital superimposition of whole mount images of corneal nerve patterns allowed several ways to test the hypothesis that nerves only enter the cornea at specific sites. These analyses revealed that nerves enter the corneal margin at many sites that are uniformly spaced around the entire cornea and uniformly spaced with respect to one another.

It has been shown previously that the density of innervation within the rodent cornea is approximately the same in the four central quadrants in the Long-Evans hooded rat [27]. In addition, computer representations of corneal innervation in the four quadrants of rat cornea whole mounts support this general conclusion [28]. In further support of this conclusion, Table 1 and Table 2 illustrate that approximately the same number of corneal nerve trunks enter each quadrant in both left and right corneas; there were no statistically significant differences in the number of nerve bundles entering specific quadrants in comparing quadrants with one another or between left and right sides. These data therefore help to explain how the density of innervation of the cornea eventually becomes the same in all four quadrants [27,28].

If, in fact, corneal innervation sites are spaced at approximately equal intervals from one another around the cornea, one possible mechanism that could produce this might involve release of neuro-repulsive factors from pioneering growth cones as they leave the pericorneal nerve ring and enter the corneal stroma during embryogenesis. Such factors would tend to keep separate bundles of growth cones well separated from one another and growing as relatively straight, centripetal radii toward the corneal center, which is the pattern observed. Growth cones of nerves following the pioneering fibers would fail to initiate new entry points because of neuro-repulsion, but could choose to respond positively to migrate along the existing pioneering fibers and thereby form fascicles/bundles, which, again, is the pattern observed. Mechanisms regulating corneal innervation remain largely uncharacterized, although they are expected to involve the glycosaminoglycan chains of stromal proteoglycans [29] and neurotrophic factors released from the corneal epithelium [30-33]. Upon entering the corneal stroma and migrating toward the corneal center for some distance, nerves in the entering bundles are then seen to bifurcate, sending some branches directly toward the corneal epithelium and some to continue onward toward the corneal center, where they turn upwards, bifurcate and innervate the epithelium [8].
A recent study of human corneal nerves using confocal microscopy has revealed that thick straight stromal nerves branch and give rise to thinner nerve fibers at various levels before they bifurcate to form dense neural plexi in the epithelial cell layer [11]. Nonetheless, that study failed to give a detailed overview of the branching pattern of the radial nerve projections. Here we analyzed E16 immunostained quail corneas using confocal microscopy to reveal that both short and long nerves exit the nerve ring at approximately the same level in the limbus, to innervate the corneal surface through the stroma. The short nerves branch immediately and rise at a steep angle in the limbus such that they enter the corneal stroma at a much higher level (nearer to Bowman’s layer) to innervate only the peripheral part of the cornea, whereas long nerves only rise slightly after exiting the nerve ring and enter the posterior part of the corneal stroma (closer to Descemet’s layer), run almost parallel to Descemet’s layer, and commence branching either shortly after entering the stroma, or in the central-peripheral part of the cornea. Some long nerves branch immediately after entering the stroma to innervate the entire corneal surface along that radius, whereas those that begin to branch in the central-peripheral area innervate only the central-peripheral and central regions of the cornea. Presence of both short and long nerves is in agreement with schematic drawings by Kitano [17] of embryonic human corneas, and by M. Ille, et al. [16] from reconstruction of ultra-thin sections of adult human corneas. However, unlike adult human corneas, where it was suggested that the long nerves only innervate the central-peripheral and central cornea [16], here we show that some of the long avian nerves innervate the entire corneal surface along a given radius. Our data suggest that avian corneal innervation, like that of the rabbit [34], is derived entirely from stromal nerves, but different from cats [35] and monkeys [36], in which the peripheral cornea is innervated by nerves from the conjunctiva.

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REFERENCES


