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Refinement of Innervation Accuracy following Initial Targeting of Peripheral Gustatory Fibers

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Abstract

During development, axons of the chorda tympani nerve navigate to fungiform papillae where they penetrate the lingual epithelium, forming a neural bud. It is not known whether or not all chorda tympani axons initially innervate fungiform papillae correctly or if mistakes are made. Using a novel approach, we quantified the accuracy with which gustatory fibers successfully innervate fungiform papillae. Immediately following initial targeting (E14.5), innervation was found to be incredibly accurate: specifically, 94% of the fungiform papillae on the tongue are innervated. A mean of five papillae per tongue were uninnervated at E14.5, and the lingual tongue surface was innervated in 17 places that lack fungiform papillae. To determine if these initial errors in papillae innervation were later refined, innervation accuracy was quantified at E16.5 and E18.5. By E16.5 only two papillae per tongue remained uninnervated. Innervation to inappropriate regions was also removed, but not until later, between E16.5 and E18.5 of development. Therefore, even though gustatory fibers initially innervate fungiform papillae accurately, some errors in targeting do occur that are then refined during later embryonic periods. It is likely that trophic interactions between gustatory neurons and developing taste epithelium allow appropriate connections to be maintained and inappropriate ones to be eliminated.

Keywords

fungiform papillae; gustatory fibers; innervation; targeting; trophic interactions; neurotrophins; taste buds

INTRODUCTION

The taste system is ideal for examining neuronal target-selection during development. Taste buds arise from oral epithelium (Barlow and Northcutt, 1995, 1997; Stone et al., 1995) and are contained within complex structures called papillae. In rodents, fungiform papillae are distributed across the rostral two-thirds of the tongue in a spatial array (Miller and Preslar, 1975) that provides discrete, predictable targets for innervating neurons. Fungiform papillae initially develop prior to and independently of sensory innervation (Farbman and Mbiene, 1991; Mbiene et al., 1997; Hall et al., 1999). However, sensory innervation is required for the maintenance of both fungiform papillae and the taste buds they contain (Nagato et al., 1995; Sollars and Bernstein, 2000; Sollars et al., 2002; Sollars, 2005). Thus, trophic interactions between fungiform papillae and gustatory neurons could shape the development of this system.

The chorda tympani nerve provides sensory innervation to fungiform taste buds. Chorda tympani fibers are capable of innervating very specific regions of the lingual epithelium

(fungiform papillae) that will eventually contain taste buds. One method by which gustatory fibers could form specific connections with developing taste buds is to hyperinnervate the lingual epithelium and then withdraw innervation from non-taste regions. However, this does not appear to occur. Initial innervation of the tongue by the chorda tympani nerve is not homogenous (Mbiene and Mistretta, 1997). Instead, the chorda tympani nerve follows a precise pathway, suggesting that neural guidance is regulated by a series of molecular cues from the environment. As chorda tympani fiber bundles approach their target, they temporarily form wide brushlike endings (Mbiene, 2004). These endings may be important for detecting cues from the epithelium just prior to target innervation. Consistent with observations suggesting that molecular cues guide chorda tympani fibers to their targets, the developing tongue produces both attractive and repulsive cues that influence the ability of gustatory axons to innervate fungiform papillae during development (Rochlin et al., 2000; Gross et al., 2003; Dillon et al., 2004).

While it is clear that gustatory axons are guided to their targets, it is not clear how many chorda tympani bundles successfully reach their correct target. That is, are fungiform papillae targeted perfectly by taste axons, or are mistakes made? It is possible that some gustatory axons innervate nontaste-bud-containing areas and then retract from these areas later during embryonic development. Likewise, taste fibers may also fail to innervate some fungiform papillae. If mistakes in innervation occur, these mistakes may be refined during later stages of embryonic development such that target innervation becomes more accurate as development proceeds.

The goals of this study were: (1) to examine the degree of accuracy with which chorda tympani fibers correctly innervate fungiform papillae immediately following initial targeting; and (2) to determine if initial mistakes in target innervation are corrected during later embryonic ages. To accomplish these goals we combined DiI-labeling with scanning electron microscopy (SEM) in the same tongues. This approach allowed us to determine whether there is a one-to-one relationship between innervation and fungiform papillae on the dorsal surface of the tongue. We found that although chorda tympani axons target fungiform papillae very accurately, a few errors in initial innervation did occur. Most of these errors were corrected during later embryonic development. This approach for quantifying targeting will prove invaluable for examining whether or not chorda tympani fibers are capable of successfully innervating their target following an experimental manipulation.

METHODS

Animals

Mice used in this study were either C57BL/6J X C3H hybrid or C57BL/6J inbred mice. Embryonic mice were obtained from mouse breedings set up just prior to the 8 h dark period. The following morning, males were removed from the cages and females were examined for plugs. This day was designated embryonic day 0.5 (E0.5). Ages were verified using morphological features for each embryo stage (Kaufman, 1995). Animals were cared for and used in accordance with guidelines of the *U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals* and *NIH Guide for the Care and Use of Laboratory Animals*.

Labeling Geniculate Ganglion with DiI

DiI-labeling has been described previously (Krimm et al., 2001). Briefly, timed-bred embryos were fixed with 4% phosphate buffered paraformaldehyde. The next day the brain and trigeminal ganglia were removed and DiI crystals (Molecular Probes, Eugene, OR) were placed on the central side of the geniculate ganglion and facial nerve. Alternatively, for some of the

embryos DiI crystals were placed in the middle ear, a location through which the chorda tympani passes on its way to the tongue. Embryos were placed between two buffer-soaked towels for 0.5–2 h, returned to 4% paraformaldehyde, and placed at 37°C for 1–12 weeks. Differences in incubation period were based on the age of the animal, because DiI transport is faster in younger than in older mice.

After incubation, the tongue was dissected, examined, and photographed using a Leica MZFL dissecting microscope and Optronics Spot-CE camera. Images were collected from two tongues from E13.5 mice, one tongue from an E14.0 mouse, five tongues from E14.5 mice, six tongues from E16.5 mice, and four tongues from E18.5 mice. Some of the labeled tongues from older embryos were cleared by placing them in 100% glycerol for several days prior to and during imaging. Optimal focal distance varied across the length of the tongue. More than one image was collected from each tongue and the images were combined so that the entire tongue surface appeared in focus. The brightness and contrast were optimized for each image. After photographing, the DiI-labeled tongues were processed for SEM.

SEM

Following DiI-labeling and imaging, tongues were rinsed in PBS, and fixed for a second time in 3.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) overnight at 4°C. Tongues were then rinsed in 0.1 M cacodylate buffer and postfixed in 1% aqueous OsO₄ for 2.0–2.5 h, washed in buffer, then dehydrated in a graded series of ethanols followed by hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate from the tongues in a dessicator overnight. Tongues were mounted on stubs, sputter-coated with gold, and examined in a scanning electron microscope (Phillips 505). Digital SEM images were captured at 130× magnification, which is high enough to distinguish fungiform from filiform papillae at all ages examined. Individual fungiform papillae were imaged at 1770× magnification.

Confocal Microscopy

Embryonic tongues were embedded in 10% gelatin solution and fixed overnight in 4% paraformaldehyde; the tongues were then sectioned at 50 μ m on a vibratome. Sections were mounted on slides, coverslipped with PBS, and viewed using an Olympus confocal microscope.

Data Analysis

Landmarks on the surface of the tongue were used to adjust the SEM tongue image size to compensate for shrinkage of the tongue during processing for SEM. The resized SEM images were overlaid onto the DiI images (Photoshop) to determine if a given papilla was innervated. Individual fungiform papillae were located and marked without knowledge of DiI distribution in the tongue. In locations where fungiform papillae were marked but there were no DiI-labeled fiber terminations nearby, papillae were designated as uninnervated. Regions where DiI-labeled fiber bundles terminated but no fungiform papillae were observed were also marked and quantified.

The total number of fungiform papillae, the number of innervated papillae, the number of uninnervated papillae, and the number of regions of innervation without viable fungiform papillae were quantified at E14.5 ($n = 4$), E16.5 ($n = 5$), and E18.5 ($n = 4$). Additional animals were used to determine the total number of fungiform papillae on the dorsal tongue surface at E14.5 ($n = 5$, total), E16.5 ($n = 6$, total), and E18.5 ($n = 4$, total). Comparisons across ages were made using analysis of variance (ANOVA), and individual means were compared using the Fisher's least-significant difference procedure. Although the alpha level was set at $p < 0.05$, the actual p values are reported.

RESULTS

In embryonic mice, the tongue almost doubled in length from E14.5 to E18.5 of development [Fig. 1(A–C)]. During this time, the fungiform papillae did not change much in size, but underwent substantial changes in morphological development [Fig. 1(D–F)]. At E14.5, the fungiform papilla is a small protuberance on the lingual surface [Fig. 1(D)], and the cells at the surface are still rounded. From E14.5 to E18.5 the cells at the papilla surface become gradually more squamous. By E18.5, filiform papillae have developed and fungiform papillae have a more adult-like morphology, although most still lack a taste pore at this age. Interestingly, there were no significant changes in the number of fungiform papillae between E14.5 and E18.5 [Fig. 1(G)]. Because papillae do not increase much in number or size between E14.5 and E18.5, the tongue increases in size primarily by adding lingual epithelium between fungiform papillae.

At E14.5, the chorda tympani nerve enters a caudal lateral point of the tongue and extends rostrally along the base of the tongue. On either side of the tongue, the nerve is located about midway between the midline and the lateral edge of the tongue. Branches exiting the main nerve extend dorsally and slightly laterally toward the epithelial surface [Fig. 2(A–C)]. These primary fiber bundles branch tremendously in a region 30–120 μm below the basal layer of the epithelium, yielding the secondary fiber bundles. Secondary fiber bundles extend parallel to the epithelial surface [Fig. 2(A–C)]. Branches exiting these bundles are perpendicular to and project toward the epithelial surface [Fig. 2(B)]. Some fiber bundles project toward fungiform papillae at this point. However, most fiber bundles have additional branches. The final branch points for fiber bundles that innervate fungiform papillae occur between 4–50 μm beneath the basal layer of the epithelium at E14.5.

At E14.5, as chorda tympani fibers penetrate the epithelial surface. They defasciculate, causing the fiber bundles to appear wider where they terminate [Fig. 2(D,E)]. These widened terminations (neural buds) can be easily identified in tongue whole-mounts [Fig. 2(F), arrows]. At E13.5 and E14.0 chorda tympani fiber bundles did not penetrate the epithelium and neural buds were not observed. Thus, gustatory fibers first penetrate the epithelium between E14.0 and E14.5 of development. At E14.5, neural buds can be aligned with the SEM images of the same tongue to determine if individual fungiform papillae are innervated. The number of innervated papillae, uninnervated papillae, and neural buds without fungiform papillae was quantifiable in overlays of DiI and SEM images at E14.5, E16.5, and E18.5 (Fig. 3).

Target innervation was highly accurate at E14.5, with an average of 79 papillae/tongue successfully innervated by chorda tympani fibers (Table 1), which is 94% of the total number of papillae. Neither the number of innervated papillae nor the total number of papillae changed from E14.5 to E18.5 of development. However, a few fungiform papillae (5 ± 1) were not innervated at E14.5 [Table 1; Fig. 3(B,F), arrow]. The number of uninnervated fungiform papillae decreased between E14.5 and E16.5 ($p < 0.03$) and remained low at E18.5 of development. As a result of this decrease more than 98% of fungiform papillae were innervated by E18.5.

Although most fiber bundles innervated fungiform papillae, some appeared to innervate regions of the tongue without fungiform papillae. An average of 17 locations in the lingual epithelium where no fungiform papillae were observed appeared to receive innervation at E14.5 [Fig. 3(F), arrowheads]. Thus, about 82% of the fiber bundles innervating the tongue successfully innervated fungiform papillae, while 18% innervated nongustatory tongue regions. This inappropriate innervation is still present at E16.5 [Fig. 3(C), arrowheads], but is reduced by E18.5 (Table 1; $p < 0.04$). Following this refinement 95% of fiber bundles correctly innervated fungiform papillae. To determine whether some of this innervation to inappropriate

regions reaches the epithelial surface, 50 μ m sections of DiI-labeled E14.5 and E16.5 tongues were examined. Some chorda tympani fiber bundles reached the epithelium, but did not penetrate it [Fig. 4(A,B), arrows]. In some locations chorda tympani innervation penetrated the epithelium where a fungiform papilla was not observed [Fig. 4(C–E)]. We did not find any innervation to nongustatory tongue regions at E18.5. This finding is consistent with the possibility that most of these inappropriate projections are withdrawn between E16.5 and E18.5 of development.

DISCUSSION

We investigated fungiform papillae and gustatory innervation at and following initial target innervation during embryonic mouse development. During targeting, chorda tympani fiber bundles are directed toward developing fungiform papillae. Just prior to target innervation, these endings temporarily expand into wider brushlike endings (Mbiene, 2004). Fungiform papillae are initially innervated by the chorda tympani nerve on E14.5 in mouse (Hall et al., 1999; Mbiene and Roberts, 2003; Mbiene, 2004). At this age, chorda tympani bundles penetrate the epithelium and form a terminal ending in the shape of a taste bud; this ending is referred to as a neural bud. This age in mouse (E14.5), is roughly equivalent to E17.0 in rat (Farbman and Mbiene, 1991), when penetration of the epithelium by chorda tympani fibers first occurs.

Two rows of fungiform placodes (early papillae) are first seen on the mouse tongue at E13.5 (Paulson et al., 1995). By E14.5 there are multiple rows of fungiform papillae (Paulson et al., 1995) and we observed that the number of fungiform papillae on the tongue does not increase after this point. Fungiform papillae differentiate substantially between when they are first innervated at E14.5 and just before birth at E18.5. The timing and types of changes that occur in the fungiform papillae of mice are similar to those of rat (Mistretta, 1972; Farbman and Mbiene, 1991), but occur roughly 2 days earlier.

Our first goal was to determine how successfully chorda tympani fiber bundles initially innervated their appropriate targets. To address this issue, we quantified innervation accuracy immediately following initial targeting of chorda tympani fibers to fungiform papillae. The vast majority of fungiform papillae (94%) are innervated by E14.5. In addition, 82% of the neural buds on the tongue were associated with fungiform papillae. Together, these findings demonstrate that initial targeting results in very accurate innervation to fungiform papillae by E14.5. However, some papillae were not innervated initially and regions of the tongue were innervated where no fungiform papillae were present. Therefore, our second goal was to determine whether or not the accuracy with which chorda tympani bundles innervate fungiform papillae improved over later stages of embryonic development. We found that the number of uninnervated papillae decreased between E14.5 and E16.5 of development such that 98% of fungiform papillae were innervated by E18.5. Likewise, the number of neural buds associated with fungiform papillae increased to 95% between E16.5 and E18.5 of development. Thus, initial innervation is followed by a period of embryonic plasticity and rearrangement.

The number of innervated locations in nongustatory epithelium decreased between E16.5 and E18.5 of development. This decrease could result from the appearance of previously undetected papillae in those locations or from the elimination of branches. If previously undetected papillae at E16.5 were more visible by E18.5, the total number of innervated fungiform papillae should have increased; however, it did not. Therefore, it is more likely that the fiber bundles innervating inappropriate lingual regions were eliminated between E16.5 and E18.5. Fiber bundles could be eliminated because they failed to innervate a region producing an adequate amount of the appropriate neurotrophic factor. The neurotrophin, brain-derived neurotrophic factor (BDNF), is present in fungiform papillae, but not in surrounding epithelium (Nosrat and Olson, 1995; Nosrat et al., 1996). Fiber bundles innervating regions without fungiform papillae

would not be exposed to BDNF, and it is possible that BDNF is required to maintain innervation. Consistent with this possibility, mice that overexpress BDNF ectopically throughout the lingual epithelium still have gustatory innervation to filiform papillae at E18.5 (Krimm et al., 2001).

It is also possible that innervation is withdrawn from inappropriate regions due to a failure of these fibers to form a synapse. In rats, synapse-like structures are first observed in fungiform taste buds between E18 and E20 (Mbiene and Farbman, 1993). Based on this finding, synapse formation between gustatory nerve fibers and taste buds should occur between E16 and E18 in mice. The development of synapses between taste buds and gustatory neurons may require either BDNF (Yee et al., 2003) or another taste epithelium specific factor. If so, synapses between gustatory fibers and nongustatory epithelium would not be possible. Perhaps during synapse formation, fiber branches forming synapses are stabilized while fiber branches that fail to form synapses are eliminated.

By E16.5, the tongue had fewer uninnervated papillae than were present earlier (E14.5), and it is possible that uninnervated papillae are eliminated between E14.5 and E16.5 of development. It is well established that during development both taste buds and fungiform papillae require innervation for their maintenance (Hosley et al., 1987; Nagato et al., 1995; Sollars and Bernstein, 2000; Sollars et al., 2002; Sollars, 2005). This trophic requirement may function to eliminate uninnervated fungiform papillae during development. However, the total number of fungiform papillae was not reduced between E14.5 and E16.5. This finding indicates that loss of fungiform papillae may not account for the decrease in uninnervated papillae. Another explanation is that uninnervated fungiform papillae become innervated between E14.5 and E16.5 of development. However, the number of innervated fungiform papillae does not increase across these ages either. This finding indicates that innervation of previously uninnervated papillae may not account for the observed decrease in uninnervated papillae. Because these two mechanisms are not mutually exclusive, some fungiform papillae that are uninnervated at E14.5 may become innervated by E16.5, while others are eliminated. In this scenario, neither the total number of papillae nor the number of uninnervated papillae would be expected to change much between E14.5 and E16.5 of embryonic development.

Initial targeting results in very accurate innervation of fungiform papillae by chorda tympani bundles. This finding illustrates the importance of guidance cues that direct gustatory fibers to fungiform papillae during development. After entering the tongue, gustatory fiber bundles change trajectories numerous times before they reach fungiform papillae (Farbman and Mbiene, 1991; Mbiene and Mistretta, 1997; Mbiene, 2004). Therefore, multiple chemoattractive and chemorepulsive cues must be present to orchestrate these changes (Tessier-Lavigne and Goodman, 1996). One such factor is probably semaphorin 3A, a chemorepulsive molecule that is expressed in developing tongue (Giger et al., 1996) and is important during both trigeminal and gustatory axon guidance (Rochlin and Farbman, 1998; Rochlin et al., 2000). Specifically, semaphorin 3A expression decreases from medial to lateral across the tongue surface and prevents premature and aberrant growth of trigeminal and gustatory fibers into the tongue midregion. In addition, as geniculate fibers near the epithelial surface, semaphorin 3A may prevent premature penetration of the epithelium (Dillon et al., 2004; Vilbig et al., 2004). The lingual epithelium also produces chemoattractants (Gross et al., 2003). Multiple chemoattractants are likely required to guide chorda tympani axons to fungiform papillae. For example, one factor, produced by the tongue, may encourage initial tongue innervation (Vilbig et al., 2004). Another factor, produced by fungiform papillae, may allow chorda tympani fibers to distinguish fungiform papillae from surrounding epithelium. Just prior to target innervation chorda tympani fibers form widened brushlike endings (Mbiene, 2004). These endings may improve the ability of chorda tympani fibers to sense and respond to molecular cues in their environment during final targeting. One candidate chemoattractant

is BDNF. It is produced in fungiform papillae but not the adjacent lingual epithelium (Nosrat and Olson, 1995; Nosrat et al., 1996, 1997), and this pattern of normal BDNF expression is important for innervation of fungiform papillae by gustatory fibers (Ringstedt et al., 1999; Krimm et al., 2001). Morphogens like sonic hedgehog or BMP4 are also expressed specifically in fungiform papillae (Hall et al., 1999, 2003; Mistretta et al., 2003; Liu et al., 2004), and could have a guidance role (Charron and Tessier-Lavigne, 2005).

The present study introduces a new approach for quantifying the accuracy with which chorda tympani fiber bundles successfully innervate fungiform papillae. Innervation accuracy immediately following targeting was compared with later embryonic ages. Immediately following initial targeting, 94% of fungiform papillae are innervated and approximately 82% of fiber bundles correctly locate fungiform papillae. Thus, most chorda tympani innervation reaches the correct location during initial targeting. Embryonic refinement of these connections results in an increase in innervation accuracy such that 98% of papillae are innervated and 95% of the neural buds correctly innervate fungiform papillae by E18.5 of development. Thus, a post-targeting refinement of innervation does improve upon the accuracy of initial targeting. Trophic interactions between taste buds and neural innervation may serve to orchestrate this refinement by maintaining innervated papillae and appropriate innervation while eliminating uninnervated papillae and inappropriate nerve branches to the epithelium. This quantitative approach for evaluating target innervation will be invaluable for examining innervation patterns following the experimental manipulation of factors that regulate gustatory neuron targeting.

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Figure 1.

As the tongue grows from E14.5 to E18.5 of development, fungiform papillae undergo substantial changes in morphology. Increases in tongue size at E14.5 (A), E16.5 (B), and E18.5 (C) are depicted using SEM. At higher magnification (1770 $\times$), developmental changes in surface papilla morphology can be observed at E14.5 (D), E16.5 (E), and E18.5 (F). No changes occur in the total number of fungiform papillae on the dorsal tongue surface between E14.5 and E18.5 of development (G). The scale bar in (C) = 600 μ m and applies to (A–C). The scale bar in (F) = 10 μ m and applies to (D–F).

Figure 2.

At E14.5 chorda tympani fibers branch extensively and change trajectory numerous times between where they enter the tongue and fungiform papillae. A side view of an entire E14.5 tongue (A) showing the initial branches exiting the chorda tympani at the tongue base, these fiber bundles branch again as they near the surface, and these branches extend parallel to the tongue surface. Fiber bundles at the tongue surface appear to project to specific locations within the lingual epithelium. Confocal images of 50 μm vibrating microtome sections (B,C) provide another view of these branching characteristics. Large fiber bundles project from the tongue base to the tongue surface (B), and nearer the epithelial surface these fiber bundles branch, extending parallel to the surface (C). Fiber bundles near the epithelial surface project to individual fungiform papillae (C). Fiber bundles defasciculate at the epithelial-lamina propria border [(D,E), arrowheads], forming a very characteristic bud-shaped termination near the

surface of the epithelium. These neural bud endings can be easily observed from the surface in tongue whole-mounts [(F), white arrows]. This dorsal surface view also clearly demonstrates the branching pattern of these fiber bundles parallel to the epithelium (F). Scale bar in (A) = 100 μm . Scale bar in (C) = 100 μm and applies to (B) and (C). Scale bar in (F) = 100 μm .

Figure 3.

SEM images of the tongue can be aligned with DiI-labeled images of the same tongue at E16.5 (A–C) and E14.5 (D–F) to quantify innervation to individual fungiform papillae. Individual fungiform papillae are identified in SEM images (A,D). While most fungiform papillae are innervated at E14.5 and E16.5, one papilla on the imaged tongue is not innervated at each age [(B,E), arrows]. In addition, there were regions on the tongue surface that appeared to be innervated where no fungiform papillae were observed [(C,F), arrowheads]. Scale bar in (C) = 100 μm and applies to (A,–C). Scale bar in (F) = 100 μm and applies to (D–F).

Figure 4.

A few chorda tympani fiber bundles project toward the epithelial surface without penetrating it at E14.5 [(A,B), arrows], and a few chorda tympani fiber bundles penetrate the epithelium in locations where no fungiform papillae are evident at E14.5 [(C), arrows] and E16.5 [(D,E), arrows]. Scale bar = 20 μ m and applies to all.

Table 1

Accuracy of Innervation to Fungiform Papillae Increases during Embryonic Development

	No of Innervated Fungiform Papillae	No of Uninnervated Fungiform Papillae	No of Innervated Regions with No Fungiform Papillae
E14.5 (<i>n</i> = 4)	79.7 ± 12.9	5.3 ± 0.9	17.5 ± 1.5
E16.5 (<i>n</i> = 5)	87.8 ± 4.4	2.0 ± 0.9*	16 ± 4.2
E18.5 (<i>n</i> = 4)	88.5 ± 4.33	1.7 ± 0.5*	4.5 ± 1.9*

Dil-SEM overlays were used to quantify the number of innervated fungiform papillae, the number of uninnervated fungiform papillae, and the number of innervated regions with no fungiform papillae. Statistical significance was determined across ages using ANOVA, and individual means were compared using the Fisher's least-significant difference procedure. Asterisks indicate significance from E14.5 at $p < 0.05$.