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Developmental Time Course of Peripheral Cross-modal Sensory Interaction of the Trigeminal and Gustatory Systems

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Abstract

Few sensory modalities appear to engage in cross-modal interactions within the peripheral nervous system, making the integrated relationship between the peripheral gustatory and trigeminal systems an ideal model for investigating cross-sensory support. The present study examined taste system anatomy following unilateral transection of the trigeminal lingual nerve (LX) while leaving the gustatory chorda tympani intact. At 10, 25, or 65 days of age, rats underwent LX with outcomes assessed following various survival times. Fungiform papillae were classified by morphological feature using surface analysis. Taste bud volumes were calculated from histological sections of the anterior tongue. Differences in papillae morphology were evident by 2 days post-transection of P10 rats and by 8 days post in P25 rats. When transected at P65, animals never exhibited statistically significant morphological changes. After LX at P10, fewer taste buds were present on the transected side following 16 and 24 days survival time and remaining taste buds were smaller than on the intact side. In P25 and P65 animals, taste bud volumes were reduced on the denervated side by 8 and 16 days post-surgery, respectively. By 50 days post-transection, taste buds of P10 animals had not recovered in size, however all observed changes in papillae morphology and taste buds subsided in P25 and P65 rats. Results indicate that LX impacts taste receptor cells and alters epithelial morphology of fungiform papillae, particularly during early development. These findings highlight dual roles for the lingual nerve in the maintenance of both gustatory and non-gustatory tissues on the anterior tongue.

Keywords

lingual nerve; chorda tympani; denervation; plasticity; sensitive period; multimodal

Sensory systems are generally thought to convey their specific and individual receptive field information without cross-modal communication at the level of the PNS. In contrast, cross-

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The authors declare no conflict of interest with the present work.

Role of authors.

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MP, AG & SS. Acquisition of data: JO, MP, AG, KA & SS. Analysis and interpretation of data: JO, MP, AG, KA & SS. Drafting of the manuscript: JO, AG, KA & SS. Critical revision of the manuscript for important intellectual content: JO & SS. Statistical analysis: JO, MP, & SS. Obtained funding: SS. Administrative, technical, and material support: SS. Study supervision: SS.

sensory interaction between differing modalities is a fundamental feature within the CNS (e.g., Angelaki and Cullen, 2008; Driver and Noesselt, 2008; Sperdin et al., 2010; Lundstrom et al., 2010; Sarko et al., 2013). Limited information exists indicating that somatosensory nerves may influence the olfactory (Frasnelli et al., 2007a, 2007b) and gustatory systems (Simon et al., 1993; Wang et al., 1995) and that the gustatory chorda tympani nerve (CT) provides support for trigeminally innervated epithelia especially in early development (Sollars et al., 2002; Sollars, 2005). The tongue serves as a particularly valuable model to explore peripheral cross-modal maintenance of morphology (Zalewski, 1981; Kinnman and Aldskogius, 1988; Oakley et al., 1993; Sollars, 2005) and functional interactions (Zalewski, 1981; Wang et al., 1995) between sensory nerves of differing modalities (i.e., the gustatory and trigeminal systems). This is in part because the tissues of each system are readily observable; on the anterior tongue the fungiform papillae and associated taste buds represent physiologically distinct structures with discrete innervation. The somatosensory lingual nerve innervates the perigemmal layer of the fungiform papillae in close apposition to the gustatory chorda tympani nerve, which transmits information from taste receptor cells within the taste bud proper (Miller, 1974), thus providing a framework to systematically manipulate innervation and examine interactions between the two sensory modes.

In spite of these separate routes of innervation, the loss of gustatory innervation via transection of the CT (CTX) in rats results in morphological changes to the lingual-innervated fungiform papillae. The extent of these changes is differentially dependent on the age at which the system is disrupted, with younger animals undergoing a near total and permanent loss of normal fungiform papillae morphology and a lack of nerve regeneration. Following CT transection in young animals (at 10 days of age; P10), taste buds degenerate by 8 days post-surgery and a majority of the fungiform papillae that persist undergo severe morphological alteration (Sollars and Bernstein, 1996; Sollars and Bernstein, 2000; Sollars et al., 2002; Sollars, 2005). CTX in adult rats does not lead to similar morphological alteration of papillae. Although reductions in taste bud volumes occur more quickly after surgery in rats transected as adults (by 2 days post-surgery following CTX at P65; Sollars, 2005), subsequent regeneration of the CT is accompanied by the re-formation of the majority of taste buds and normalized fungiform papillae (St. John et al., 1995; Montavon et al., 1996; Kopka et al., 2000; Sollars, 2005).

Combined denervation of the CT and lingual nerves leads to more severe changes than after transection of the CT nerve alone (Whitehead et al., 1987; Hard af Segerstad et al., 1989; Ganchrow and Ganchrow, 1989), thus implicating an interactive nature of the two nerves in the maintenance of taste buds and papillae. Additionally, the persistence of some fungiform taste buds following chronic CTX in adult rats has been suggested to be due to the presence of the lingual nerve; taste buds remaining after CTX appear to be maintained by lingual nerve fibers (Kinnman and Aldskogius, 1988). The presence of peptide-expressing lingual nerve fibers in close proximity to taste cells (Finger, 1986; Kinnman and Aldskogius, 1991) and functional evidence of trigeminal mediation of gustatory nerve responses (Wang et al., 1995) further support an intriguing potential interdependence between gustatory and non-gustatory structures in the peripheral taste system.

The present study examined the effects of unilateral transection of the lingual nerve proper (LX) while leaving the CT intact. Ages at the time of surgery were P10, P25, and P65 representing neonatal, juvenile, and adult maturational stages of the taste system. This provided a method to study the ability of the CT to maintain taste buds and papillae morphology across development following the loss of lingual nerve innervation. Determination of the time course of LX effects was accomplished by examining the morphology of fungiform papillae at 2, 8, 16 or 50 days post-transection for each postnatal surgical age. Based on the findings of the morphological study, taste bud volumes were assessed at 2, 8, 16, 24 or 50 days post-transection. Changes to taste buds following LX would suggest the existence of a synergistic relationship between two anatomically distinct peripheral systems, both of which receive innervation from different peripheral ganglia and carry out independent functional roles in the peripheral nervous system.

METHODS

Animals

Female Sprague-Dawley rats ($N = 135$) were obtained from a breeding colony at the University of Nebraska at Omaha. Day of birth was designated P0 and litters were culled to a maximum of 12 rats per litter. Pups were weaned at P25 and maintained ad libitum on standard rat chow (Teklad) and water. Each surgical condition was comprised of animals from at least two different litters. All procedures were carried out under the approval of the University of Nebraska at Omaha Institutional Animal Care and Use Committee and in full accordance with NIH guidelines.

Surgery

At 10, 25, or 65 days of age (as neonate, juvenile, or adult, respectively), unilateral LX was performed. The taste system of rats undergoes a period of rapid development during the first postnatal weeks and becomes functionally mature around 40 days of age (Krimm and Hill, 1998), thus the surgical ages used here represent varied developmental stages and are consistent with previous investigations (e.g., Krimm and Hill, 1998; Sollars, 2005). For the surgery, rats were anesthetized with methohexital sodium (Brevital®; 50 mg/kg, i.p.) and an incision was made on the ventromedial portion of the neck. On the right side, the digastricus and masseter muscles were dissected using microfine Dumont forceps until the lingual nerve was visualized, then traced to its point of bifurcation with the CT nerve. The lingual nerve was cut proximal to its juncture with the CT, as close as possible to the foramen ovale, while keeping the CT intact. The complete division of the sectioned ends of the lingual nerve and the intact CT were visualized to verify success of the surgical procedure.

Procedures were conducted by a researcher experienced in both lingual and CT transections, and particular attention was given to avoid disturbing the CT during the LX procedure. The contralateral side of the tongue served as intact tissue for comparison, with identical surgical procedures performed on the left side, but the nerves were left intact following visualization. Taste bud volumes from the intact sides of the tongue reported here were directly comparable and often reliably smaller than measurements from the intact sides of similarly aged CTX animals (Sollars, 2005) and volumes observed in nonsurgical animals used in a

behavioral study (unpublished data). Following animal sacrifice, the CT was visualized in several animals to ensure that only the lingual nerve was impacted during transection. No indication of CT damage was observed during these procedures.

Papillae Morphology

Following 2, 8, 16, or 50 days survival post-transection, rats (N = 5/condition) were given an i.p. overdose of ketamine/xylazine and perfused with a modified Krebs solution, followed by 8% paraformaldehyde. Following perfusion, the anterior tongue was removed from the oral cavity by severing the tongue just posterior to the intermolar eminence, and the tissue was postfixed in 8% paraformaldehyde for at least one week. The ventral side of the tongue was cut off and remaining muscle layers were scraped away using a scalpel, leaving only the dorsal epithelium intact. The dorsal epithelium was placed into a 0.5% methylene blue solution, dried, and secured between two microscope slides.

Fungiform papillae were visualized and counted using a combination of brightfield and phase contrast microscopy. Observed changes in papillae morphology were delineated, based on the well-characterized categories of “pore,” “no pore,” or “filiform-like” (Parks and Whitehead, 1998; Sollars and Bernstein, 2000; Sollars et al., 2002; Sollars, 2005). Figure 1 provides illustrative examples of each of these morphological types. Using Neurolucida (MBF Bioscience) software, morphological categories were digitally mapped across the full extent of the anterior tongue, see Figure 2. Mapping and category classifications were conducted while ensuring that the investigator was unaware of the animal condition.

Taste Bud Morphology

Given findings from the surface analysis, an additional post-surgical age (24 days post) was included in the analysis of taste bud volumes, resulting in survival time points of 2, 8, 16, 24 or 50 days. To ensure a substantive number of taste buds were included in the analysis, an additional rat per group (N = 3) was used as compared to previous reports of taste bud volume (Sollars et al., 2002; Sollars, 2005) and consistent with previous reports (Shuler et al., 2004; Hendricks et al., 2004; Guagliardo and Hill, 2007). Following perfusion procedures identical to those stated above, tongues were removed, stored in 8% paraformaldehyde for at least one week and then cryoprotected overnight in a 40% sucrose solution. The rostral-most 2 mm of each tongue was removed and discarded prior to sectioning to avoid skewing volume measurements, since taste buds at the tip of the tongue tend to be significantly smaller, with more variability between buds, than those on the mid-regions of the tongue (Krimm and Hill, 1998). This procedure is consistent with previous work (Krimm and Hill, 1999; Hendricks et al., 2004, Sollars et al., 2002; Sollars, 2005). Serial 10 μm coronal sections were collected through the subsequent 2 mm of tissue using a cryostat, and tissue was stained with hematoxylin and eosin.

Findings from the surface analysis indicated that the effects of LX were evident across the entire fungiform field (see Figure 2), thus removal of the tongue tip allows for an accurate representation of volume measurements. Neurolucida software was used to digitally trace the perimeter of each taste bud. Border cells (evident by differential coloration following

staining) were included in volume measurements in order to be consistent with previous reports (for example of tracing area, see Figure 1; Krimm and Hill, 1998; Sollars et al., 2002; Sollars, 2005). Total volume of each taste bud was calculated from the area defined by perimeter tracings across sections and multiplying this value by 10 (i.e., the section thickness). All morphological analyses were conducted by a trained observer, blind to treatment condition.

To visualize if remaining taste receptor cells on the LX sides of the tongue appeared disrupted similar to what is noted after neonatal CTX (Sollars et al., 2002) qualitative analysis of cytokeratin-19 (CK-19) stained tissue was conducted in rats from a subset of surgical conditions, since CK-19 needs to be performed on tissue that has not been perfused. For these analyses animals were sacrificed by i.p. injection of ketamine/xylazine and tongue tissue was removed, rinsed briefly in PBS and dried. Tongue tissue was frozen rapidly and then stored at −80°C until sectioning. Sections 10 μm thick were obtained from the tongue beginning 2 mm from anterior tongue tip (from the same portion of the tongue analyzed for volume measurement). Mounted tissue sections were treated with monoclonal anti cytokeratin-19 (1:1600; Sigma) and Alexa Fluor 546 (1: 800; Invitrogen). The presence of cytokeratin-19 positive cells was noted on LX and control sides of the tongue.

Data Analysis

Using analysis of variance (ANOVA), papillae types were compared between the experimental (LX) and control (intact) sides of the tongue. Bonferroni post hoc tests were conducted to assess differences at each surgical age for the various survival times (e.g., tongues were compared across sides in animals transected at either P10, P25 or P65 at 2 days post-surgery; Krimm and Hill, 1998; Sollars et al., 2002; Sollars, 2005). The emphasis of the analyses was on the effects of LX at each maturational age as observed after a defined survival period. Grouping the data as a condition of survival time allowed comparisons between the surgical side of the tongues given both the maturational development at the time of LX and the time post-surgery. Concurrent with the volume measurements, taste bud frequency was qualitatively assessed in these animals.

ANOVA was also used to assess changes in taste bud volume following surgery at 10, 25 or 65 days of age. Following a significant ANOVA, planned post hoc comparisons using independent sample t-tests were conducted between the LX and intact sides of the tongue for animals of the same age at sacrifice (e.g., the LX and intact sides of the tongues in all P10 animals sacrificed at 2 days post-surgery). The alpha level was set at $p < 0.05$, for all analyses.

RESULTS

Following LX, both taste bud volumes and papillae morphology were altered, although this finding varied dependent on the age at which transection was performed. These findings suggest a significant cross-modal relationship between the taste and trigeminal systems, in that loss of neural input from the lingual nerve proper interferes with the maintenance of both non-gustatory (i.e., fungiform papillae) and gustatory (i.e., fungiform taste buds)

tissues, even though the CT remains intact. Furthermore, these effects are particularly pronounced during early development.

Papillae Number and Morphology

Overall—Findings from the surface analysis suggest that changes to papillae morphology as a result of LX are highly dependent on the age at which neural loss occurs. Considerable effects occurred following transection early in development, with no significant changes in animals cut at P65. Furthermore, the observed alterations of papillae in animals transected at P10 or P25 dissipate sometime between 16 and 50 days post-surgery. See Table 1 for mean frequency (± SEM) and percentage of total papillae by each morphological type.

Total Papillae Numbers—The total number of papillae (i.e., combined numbers of papillae with pore, no pore, and filiform-like) did not significantly differ between intact and LX sides in any of the groups examined (all p's > 0.05). Similarly, the total number of observable papillae on the intact side did not significantly differ by age (all p's > 0.05). Surface analysis results for papillae with pore, no pore, and filiform-like papillae across groups are depicted in Figure 3.

2 Days Post-Surgery—Overall, results at 2 days post-surgery indicate that neonatal loss of lingual input quickly results in negative outcomes for the maintenance of typical papillae morphology, but this effect is not apparent in older animals. ***Filiform-like.*** The first filiform-like papillae were observed on the cut side of the tongues following 2 days survival time in P10 LX rats and this was a statistically significant increase, as no such structures were observed on the intact side ($p = 0.03$). None of the rats transected at P25 or P65 had any filiform-like papillae at this early time point. As a result, this finding also represented a significant difference between the cut sides of the tongues of P10 animals compared to those transected at P25 or P65 ($p = 0.03$, for each). ***No Pore.*** There was a significant increase in the number of no pore papillae on the denervated compared to the intact sides of the tongues in P10 animals ($p = 0.003$) by 2 days post-surgery. No other group differed significantly in the number of no pore papillae at this time point. ***Pore.*** The overall ANOVA for the number of papillae with pores showed significant differences ($F_{(1,29)} = 13.83$, $p = 0.001$). However, when examined with post hoc tests, no significant differences were noted between the cut and intact sides of the tongue, regardless of age of surgery. Similarly, no meaningful significant differences were found between the transected sides of the tongue between animals of the various surgical ages.

8 Days Post-Surgery—Results found 8 days after surgery suggest that changes in morphology following LX is not a unique characteristic of neonatal surgery, as juveniles are likewise impacted following nerve loss. Adult papillae were not significantly altered by LX at this time point post-surgery, a result which highlights the importance of developmental maturity in the role of the lingual and CT nerve interaction. ***Filiform-like.*** A few filiform-like papillae were observed 8 days after surgery on the cut sides following transection on P10, P25 and P65 and the overall ANOVA was significant ($F_{(1,28)} = 7.70$, $p = 0.01$). However, post hoc analysis of these findings resulted in no significant differences between sides of the tongue in P10, P25 or P65 rats, likely due to the fact that filiform-like papillae

were disparately present within animals in each surgical group at this time point. Similarly, when looking at the denervated side of the tongues, there were no significant differences across surgical ages. ***No Pore.*** There were a greater number of no pore papillae ($F_{(1,28)}$ = 28.61, $p < 0.001$) on the transected sides of the tongue in both P10 ($p = 0.05$) and P25 animals ($p = 0.001$) compared to the intact side of the tongue when observed 8 days after LX. No significant differences in the number of no pore papillae were found for P65 animals. Additionally, the denervated side of the tongues did not significantly differ in the number of no pore papillae when P10, P25 and P65 were compared to each other. ***Pore.*** Significant variation of papillae with pores was present at this time point ($F_{(1, 28)} = 20.07$, $p < 0.001$), but these results were non-significant in post hoc testing across both surgical conditions and differing ages.

16 Days Post-Surgery—A distinct relationship between age at nerve loss and severity of changes in papillae morphology was also evident at this time post-transection, with younger animals impacted considerably more than juveniles or adults. ***Filiform-like.*** Filiform-like papillae were found significantly more frequently ($F_{(1, 28)} = 33.94$, $p < 0.001$) on the cut side of the tongue by 16 days post-surgery in P10 transected animals ($p < 0.001$). Every animal transected at P25 or P65 also had some filiform-like papillae on the LX sides of the tongue at this time, though there were no significant differences from the intact side at this time point for the juvenile and adult animals. ***No Pore.*** A significant increase ($F_{(1, 28)} = 29.14$, $p < 0.001$) in the number of no pore papillae was observed for rats cut at P10 ($p < 0.001$). No such differences were found for animals transected at P25 or P65. Additionally, when the transected sides of the tongue were compared across development, P10 animals had significantly more no pore papillae than P25 ($p = 0.006$) and P65 animals ($p = 0.001$). No significant differences were found between the cut sides of the P25 or P65 surgical ages. The number of papillae with pores was also reduced on the cut versus the intact side at 16 days post-surgery ($F_{(1, 28)} = 44.69$, $p < 0.001$). Post hoc analysis revealed the differences occurred following transection at P10 ($p < 0.001$) and P25 ($p = 0.012$). ***Pore.*** There were significantly fewer papillae with pores in P10 animals than P65 animals when the LX sides of the tongue were compared to one another ($p = 0.046$). No significant differences in pore number were found when the LX sides were compared to P25 animals.

50 Days Post-Surgery—LX and intact sides of the tongue did not differ in numbers of filiform-like papillae ($F_{(1,26)} = .63$, $p > 0.05$), no pore papillae ($F_{(1, 26)} = 2.27$, $p > 0.05$), nor papillae with pore ($F_{(1, 26)} = 3.28$, $p > 0.05$).

In total, these findings suggest the loss and subsequent return of lingual innervation, as even the most detrimentally impacted animals (those that had LX at P10) exhibit a return to typical morphology by the latest time point. For the taste bud volume analysis, we added an additional post-surgical age (24 days) to help delineate the time recovery occurs.

Taste Bud Volumes

Overall—Analyses indicated that loss of the lingual nerve leads to disruptions in fungiform taste buds on the anterior tongue, however the CT is capable of some taste bud support in the absence of the lingual nerve. LX at an early age increases both the severity and duration of

reduced taste bud volumes. Comparisons of the effect of surgery on taste bud volumes (LX versus intact sides of the tongue) were significant for each surgical age; P10 $F_{(1,325)} = 60.96$, $p < 0.001$; P25 $F_{(1,423)} = 26.10$, $p < 0.001$; P65 $F_{(1,380)} = 12.04$, $p = .001$. Figures 4–8 illustrate the relative distributions of taste bud volumes across all treatment conditions. Qualitative analysis of CK-19 stained tissue showed that the receptor cells within remaining taste buds maintained the typical fusiform shape (see Figure 1), and did not appear disrupted as is seen after neonatal CTX (Sollars et al., 2002). Many empty papillae were observed in P10 animals, and reduced cell numbers were apparent in taste buds of both P10 and P25 animals at 8 and 16 days post-surgery. All observed taste buds contained at least some CK-19 positive cells.

2 Days Post-Surgery—Reduced taste bud volume on the LX side of the tongues was found 2 days after surgery in animals that received LX at P10 ($p = 0.011$) or P25 ($p = 0.018$) when compared to the intact side (see Figure 4). P65 animals had no differences in taste bud volumes across the tongue at this early time point.

8 Days Post-Surgery—Analysis at 8 days post-surgery showed significant reduction in taste bud volumes on the cut side of the tongues as compared to the intact side, following transection at P10 ($p < 0.001$) or P25 ($p < 0.001$). P65 animals had no significant differences in taste bud volumes on the cut versus intact sides of the tongue (see Figure 5). Thus, the sufficiency of the CT to support taste buds in the absence of the lingual nerve appears diminished in neonatal and juvenile animals, but this ability is retained in adult rats.

16 Days Post-Surgery—There was a considerable loss in the observed numbers of taste buds within the P10 animals (across 3 animals: on the LX side $N = 13$, control side $N = 48$) and remaining taste buds on the cut side were significantly smaller than those on the intact side ($p = 0.005$). Examination following 16 days survival time also revealed a significant reduction in taste bud volume on the LX side as compared to the intact side in both P25 ($p = 0.022$) and P65 animals ($p = 0.014$). However, differences in the number of taste buds were not noted in juvenile or adult rats. These results indicate that LX at any age interferes with taste bud maintenance, but these effects are profoundly more severe following lingual nerve loss at P10. See Figure 6 for taste bud volume distributions.

24 Days Post-Surgery—By this time point, there were only a total of 8 taste buds found on the transected side of the tongues across the 3 neonatally transected animals as compared to 32 taste buds on the intact side. The few remaining taste buds were significantly smaller than those on the intact side ($p < 0.001$). Interestingly, by 24 days after surgery, taste bud volumes normalized across sides of the tongue in P25 animals. However, taste buds on the transected side remained significantly smaller than the intact side in P65 animals ($p = 0.006$; Figure 7). It appears that both the onset and recovery of effects are delayed in animals transected at P65.

50 Days Post-Surgery—By 50 days post-surgery, P10 animals demonstrated the return of taste buds in numbers equal to that of the uncut side, however taste bud volumes remained significantly smaller on the LX side of the tongue ($p = 0.002$; Figure 8). Taste bud volumes were similar between surgical conditions in P25 and P65 transected animals at 50 days post.

It remains to be seen if the losses in taste bud volume following neonatal LX persist akin to the permanent absence of fungiform taste buds after CT transection at 10 days of age (Sollars, 2005).

Summary—Similar to the findings of the surface analysis, LX leads to changes in the morphology of fungiform taste buds. In juvenile and adult animals, the CT appears capable of supporting taste receptor cells in the absence of the lingual nerve, while the CT in neonatal rats appears to need the support of the lingual nerve to maintain taste receptor cells to an appreciable degree. Strikingly, even adult animals underwent reductions in taste bud volume as a result of lingual nerve loss. While the changes were more severe and longer lasting for the youngest animals, these findings suggest the lingual nerve is important for fungiform taste receptor maintenance regardless of age of injury.

DISCUSSION

The results of the present study provide clear evidence for peripheral cross-modal sensory support between the gustatory and trigeminal systems with an interdependence predicated on factors which vary based on the age of the animal at time of surgery. It is highly unusual to observe naturally occurring cross-sensory interactions at the level of the periphery, making the relationship seen here between the morphologically discrete gustatory and somatosensory systems all the more striking. Further, we show evidence for a developmental sensitive period after LX similar to that found in previous CTX research (Sollars, 2005). The regenerative capacity of taste organs and the neural independence of each of the sensory systems become more pronounced with maturation.

The CT alone, in the absence of the lingual nerve, appears to have a greater ability to support taste buds in adult rats than in neonatal and juvenile rats. The integrity of the somatosensory lingual nerve has a vital impact on the maintenance of fungiform taste buds in rats, even though it has no synaptic contact with the taste cells within those buds (Beidler and Smallman, 1965; Lopez and Krimm, 2006). Additionally, after LX (leaving the CT intact), the remaining CT innervation appears sufficient to maintain most of the fungiform papillae although the CT normally terminates solely within the taste bud (Miller, 1974). When LX occurs in neonatal rats, the majority of taste buds ipsilateral to LX are temporarily lost and the remaining buds are typically smaller than those on the intact side of the tongue. The impact of LX is reduced in juvenile and adult rats as a larger number of taste buds remain following the loss of lingual nerve input, though transient reductions in taste bud size are observed.

Regardless of surgical type (i.e., LX or CTX), maturational status impacts the anatomical effects with greater changes following neonatal transection than after transection in adulthood (Sollars et al., 2002; Sollars, 2005). For example, LX surgery at P10 results in a dramatic loss of fungiform taste buds found on the transected side of the tongue at 16 and 24 days after surgery, while such reductions were not observed after LX at P25 or P65. Developmental maturation was also apparent in the regenerative capacity of taste buds; reductions were transient in juvenile and adult animals but volumes on the LX side of the tongue in P10 animals remained lowered during the duration of the study (to at least 50 days

post-surgery). Additional research will be needed to examine if the reductions are permanent, as is seen following neonatal CTX (Sollars and Bernstein, 2002; Sollars, 2005).

Throughout the lifespan of the rat, a highly dynamic environment exists in which taste receptors cells turn over approximately every 10 days (Beidler and Smallman, 1965; Hendricks et al, 2004) with more rapid proliferation during early postnatal development (Hendricks et al, 2004). The heightened proliferation rates appear to parallel the time course of neural dependence in postnatal and early adult rats. Papillae formation begins prior to neural innervation during embryonic development (Farbman and Mbiene, 1991; Mbiene et al., 1997) and a similar occurrence has been suggested for embryonic taste bud development (Ito et al., 2010). Age-related shifts in the importance of neural innervation occur wherein both fungiform taste buds and papillae appear to become dependent on nerves for maintenance in the early postnatal ages (Mistretta et al., 1999; Sollars et al, 2002). This is followed by a reduced need for support by papillae in adulthood (Hard af Segerstad et al., 1989; St. John et al., 1995; Sollars, 2005) and an apparent return to dependence at the end of the lifespan (He et al., 2012). While previous studies have focused on transections of the CT or combined chorda-lingual nerves, the present report is the first to describe the developmental dependence of early postnatal taste buds on the presence of the lingual nerve proper. As such, the role of the lingual nerve in the regulation of taste cell dynamics is not currently known. LX during this time of particularly intense neural dependence may disrupt the mechanism of increased taste receptor cell proliferation and lead to loss of taste buds.

As taste nerve transection is known to alter the amount of neurotrophic factors found in peripheral taste tissue (Yee et al., 2005), the impact of LX on taste buds may be related to an interruption in the availability of supportive circulating factors normally delivered via the lingual nerve. The interplay between neural dependence and trophic support of taste receptor cells has been demonstrated in circumvallate papillae. Following glossopharyngeal nerve crush in adult mice, Sonic hedgehog (Shh) expression decreased within 6 hours and was subsequently implicated in the return of taste buds following reinnervation (Miura et al., 2004). In the anterior tongue, neurotrophins such as NT-3 have previously been implicated in the cross-modal support of gustatory structures (Nosrat et al., 1997) and loss of such support may explain the effects following LX seen here.

The current study further expands our knowledge of developmentally mediated neural reliance by demonstrating that while the lingual nerve is canonically responsible for fungiform papillae functionality, its presence is not strictly required for papillae maintenance. Despite the observed epithelial changes, at least some normal papillae with pores were maintained following LX at any age. Filiform-like papillae were seen by 2 days after LX at 10 days of age and some filiform-like papillae were evident across all ages by 8 days post-surgery, suggesting that epithelial maintenance of fungiform papillae is disrupted following neural denervation. Epithelial alterations (e.g., the keratinized filiform-like papillae; Parks and Whitehead, 1998) may center on changes in availability of epidermal growth factor (EGF) or receptor expression. EGF is found in the dorsal epithelium of fungiform papillae and has been linked to the maintenance of both taste bud and papillae morphology (Morris-Wiman et al., 2000). EGFR−/− mice develop a high number of filiform-like papillae despite retained neural innervation (Sun and Oakley, 2002). Similar to

what was found in the present study, the knockout mice developed the same overall numbers of fungiform papillae as wild type animals, implicating EGF in proper papillae maintenance beyond the initial development of these structures.

Rats that received LX at any age eventually experience a recovery from the effects of lingual denervation on papillae morphology, as these changes were transient—if slightly delayed—in even the youngest animals. This is in sharp contrast to the failure of papillae to return after neonatal CTX regardless of recovery time (Sollars and Bernstein, 2000). For example, LX at 10 days of age results in equal numbers of papillae with pores between intact and cut sides by 50 days after surgery, whereas the transected side of the tongue in P10 CTX rats has a 62% reduction in identifiable papillae compared to the intact side following 50 days recovery time (Sollars, 2005). Similarly, no filiform-like papillae remain 50 days after LX at P10, while in neonatal CTX rats the majority of remaining papillae are filiform-like (Sollars, 2005). Furthermore, at no time after LX did animals have significantly different numbers of total papillae regardless of surgical age, however, CTX results in significant loss in the overall number of identifiable papillae in both 10-day and 25-day surgical animals.

It is likely the observed progression toward recovery of taste buds and papillae morphology after LX is the result of lingual nerve regeneration, though the present study was not designed to assess this directly. Strikingly, it also appears that lingual regeneration occurs even following LX at early postnatal ages. This is in sharp contrast to what is observed after neonatal CTX. While recovery following adult CTX is accompanied by regeneration of the CT at around 35 days after surgery (St. John et al., 1995), there appears to be a permanent failure of the CT to regenerate after CTX in early postnatal ages (Sollars & Bernstein, 2000; Sollars et al., 2002; Martin and Sollars, 2015). Reports following inadvertent damage to the lingual nerve during dental surgeries provide evidence of the nerve's regenerative capacity in human adults (Holland, 1996; Iro et al., 2005). Regeneration and functional recovery is typically observed within 3 months following this type of nerve injury (Holland, 1996). A variety of clinical interventions including nerve grafts, electrical stimulation, and neurotrophic therapies have also been shown to encourage peripheral nerve regeneration when spontaneous recovery fails to occur (Iro et al., 2005; English et al., 2014).

The current findings support a bidirectional, multimodal interaction between the taste and trigeminal nerves in supporting their associated structures and indicate that this relationship is mediated by developmental factors. While co-dependence between these otherwise distinct sensory circuits has been previously established (Kinnman and Aldskogius, 1988; Sollars et al., 2002; Sollars, 2005), we demonstrate here that gustatory and somatosensory anatomy is impacted by targeted loss of the lingual nerve, despite continued CT innervation. Given the effects of LX on taste buds, it is likely that gustatory function is also disturbed. Whether multimodal function and morphological changes also occur centrally after LX is not yet known. Sensitive periods, such as we observe here for lingual nerve and CT maintenance of tissues in the oral cavity, have been important to understanding the limits and extent of plasticity in both peripheral and central nervous systems (e.g., Hubel and Wiesel, 1970; Blakemore and Van Sluythers, 1973; Hosley et al., 1987; Farbman et al., 1988; Hashisaki and Rubel, 1989; Sollars, 2005; Erzurumlu and Gaspar, 2013, for review). Our discovery of a sensitive period for the bidirectional nature of multi-sensory support and

maintenance may have implications for other systems, especially in which two or more sensory nerves are in close anatomical apposition.

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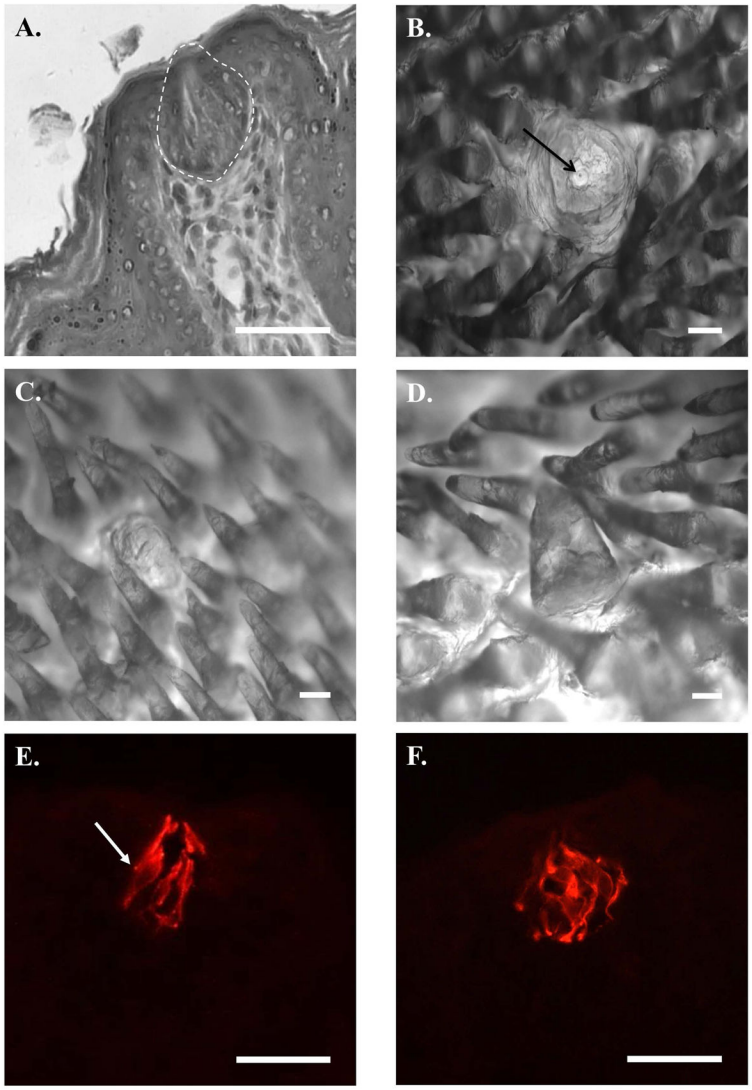

Figure 1.

(A) Photomicrograph of 10 μm tongue section illustrating a normal fungiform papillae with taste bud outlined. Surface images of tongue tissue illustrating (B) papillae with pore (pore area indicated by arrow), (C) no pore papillae, and (D) filiform-like papillae morphology. Cytokeratin-19 (CK-19) positive cells within fungiform papillae from the cut and intact sides of the tongue in an animal 16 days after surgery at P10. (E) Taste bud with few CK-19 positive cells on the lingual denervated side of the tongue. Solid arrow denotes a single taste receptor cell, which maintains typical fusiform shape despite loss of lingual input. (F) Taste bud from intact side of the tongue, with full complement of taste receptor cells. Scale bar = 50 μm.

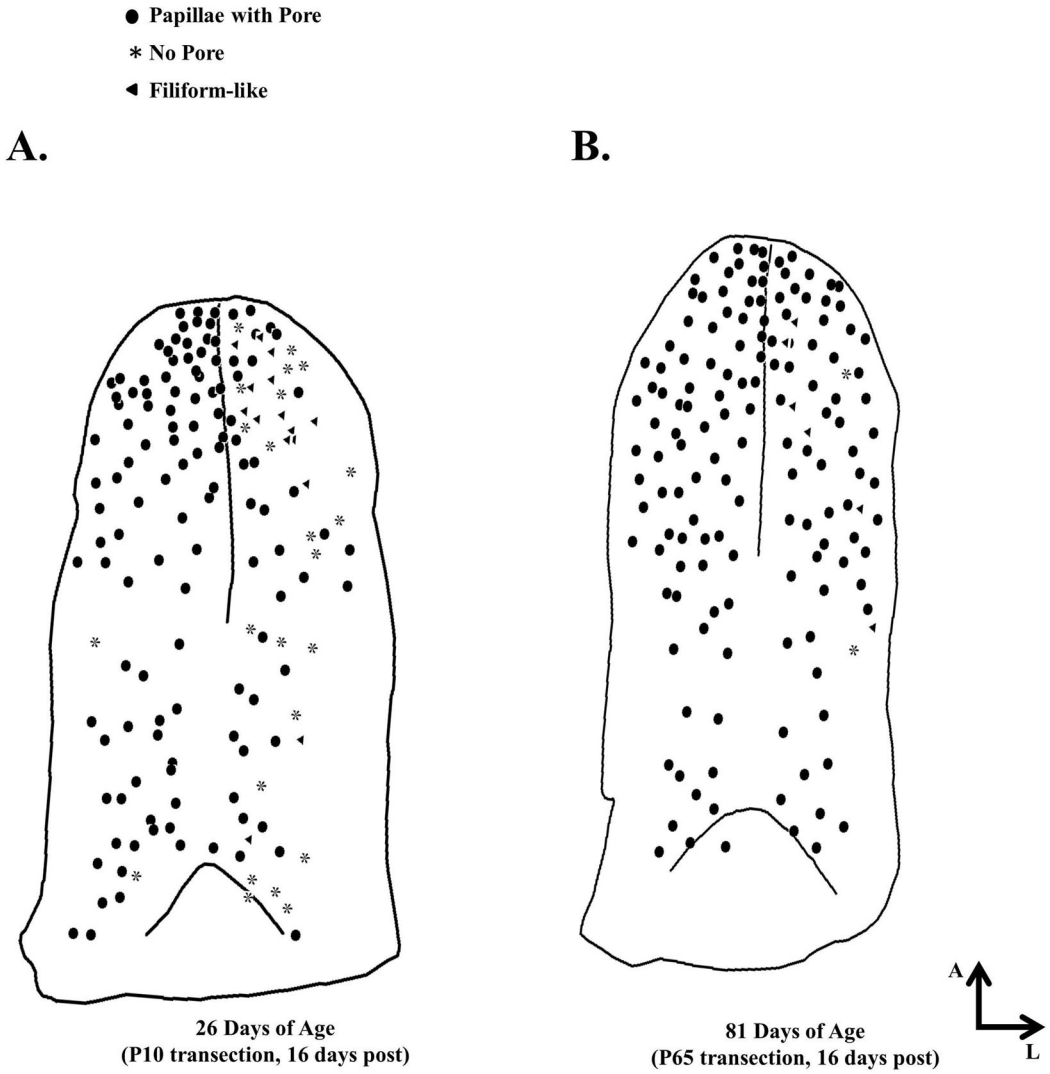

Figure 2.

Example of surface analysis in animals 16 days after lingual transection on the right side of the tongue at (A) P10 or (B) P65. The left side of each tongue served as control tissue for each animal. Circles indicate fungiform papillae with pore, asterisks denote no pore papillae and triangles denote filiform-like papillae. Vertical, anterior center line indicates midline of the tongue and horizontal, posterior line represents the intermolar eminence. A- anterior, L- lateral.

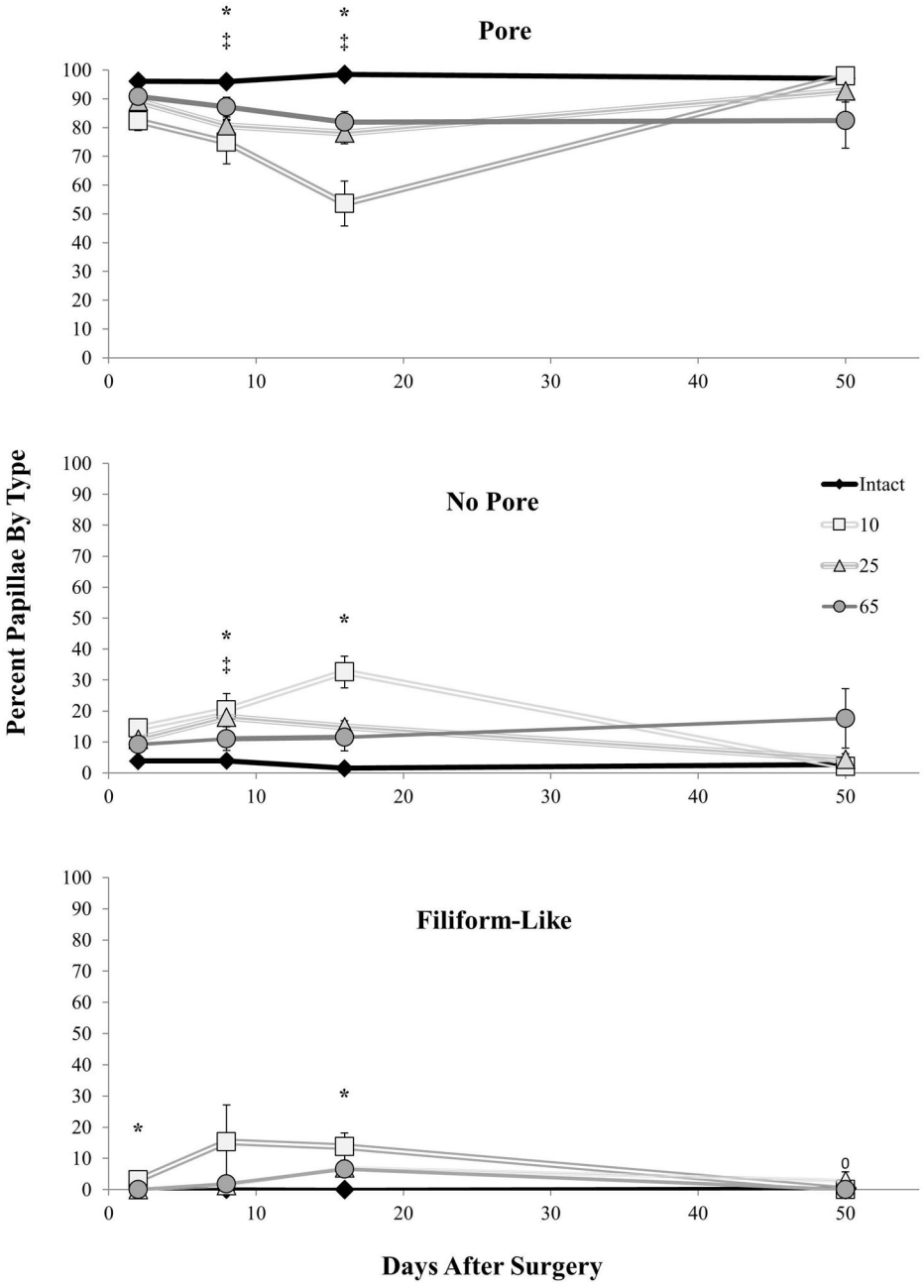

Figure 3.

Papillae surface analysis by morphological type, across all surgical ages at 2, 8, 16 or 50 days post-transection. To capture differences within groups, the frequency of each papillae type was calculated as a percentage of the total number of observed papillae, rather than raw frequency. No significant differences across any of the morphological classifications were found between intact sides of P10, P25 or P65 animals so, for illustrative purposes, intact sides were collapsed across surgical ages for pore ($F_{(1, 9)} = 0.002$, $p > 0.05$), no pore ($F_{(1, 9)} = .31$, $p > 0.05$), or filiform-like counts ($F_{(1, 9)} = .51$, $p > 0.05$). * denotes significant

differences between intact and P10 animals. ‡ denotes significant differences between intact and P25 animals.

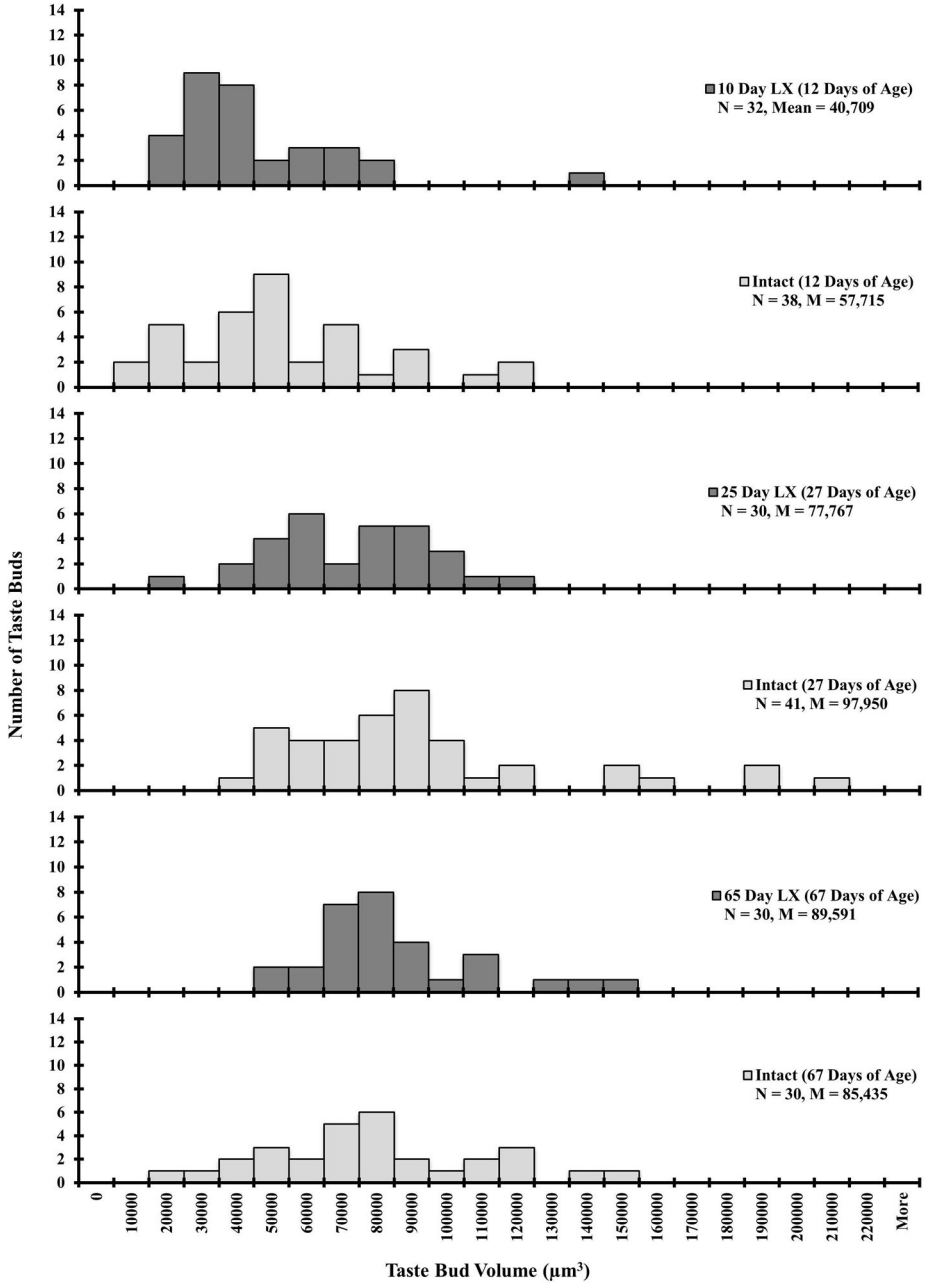

Figure 4.

LX was performed when animals were 10, 25 or 65 days of age and tongues were examined 2 days after surgery. Histograms depict the number of taste buds per volume range. Volumes were assessed on both the LX side and control (intact) side of the tongue, and included all taste buds within the standardized area.

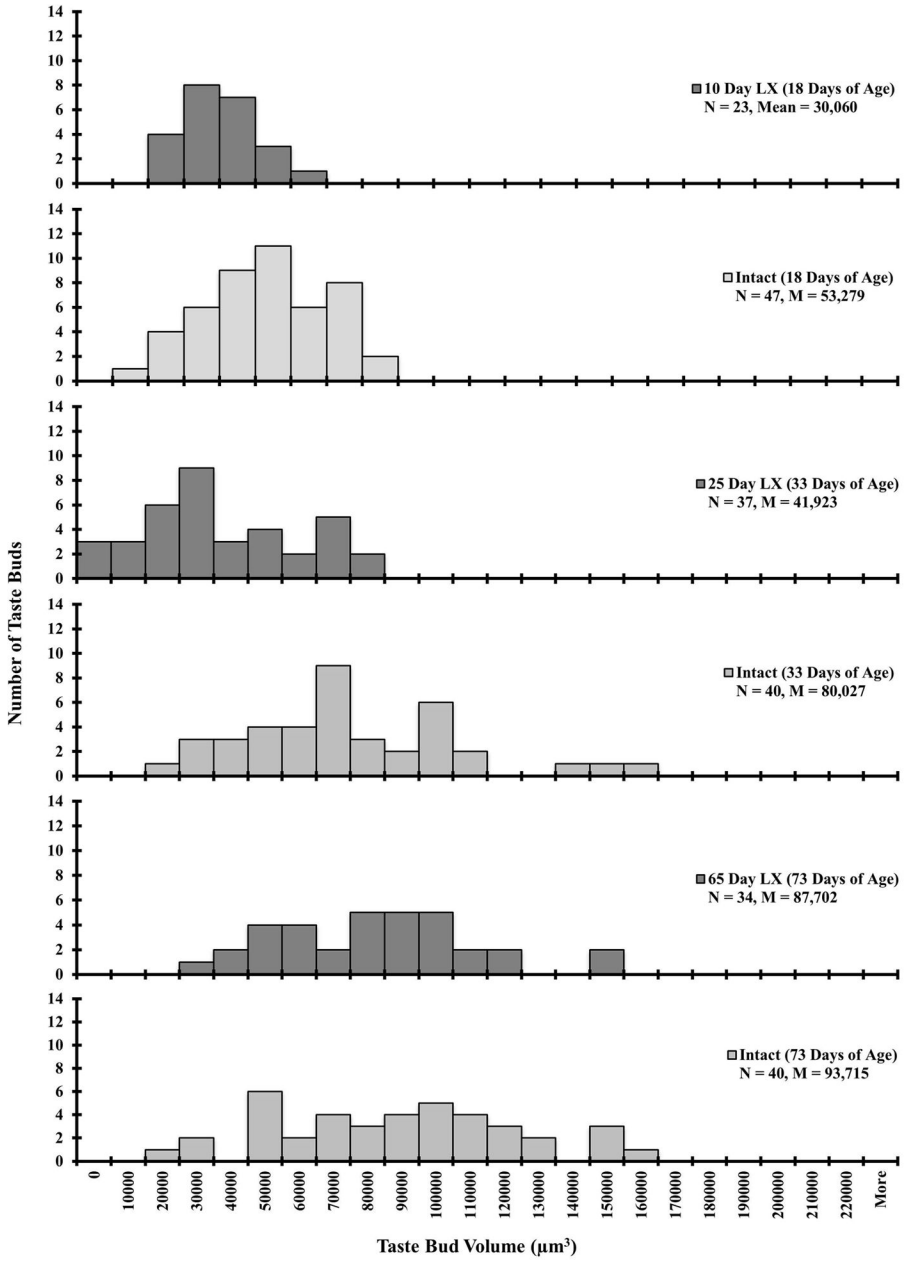

Figure 5.

LX at 10, 25 or 65 days of age and tongues were examined 8 days after surgery. The number of taste buds per volume range is depicted. Volumes on both the LX side and control (intact) side of the tongue are included from taste buds in the standardized area.

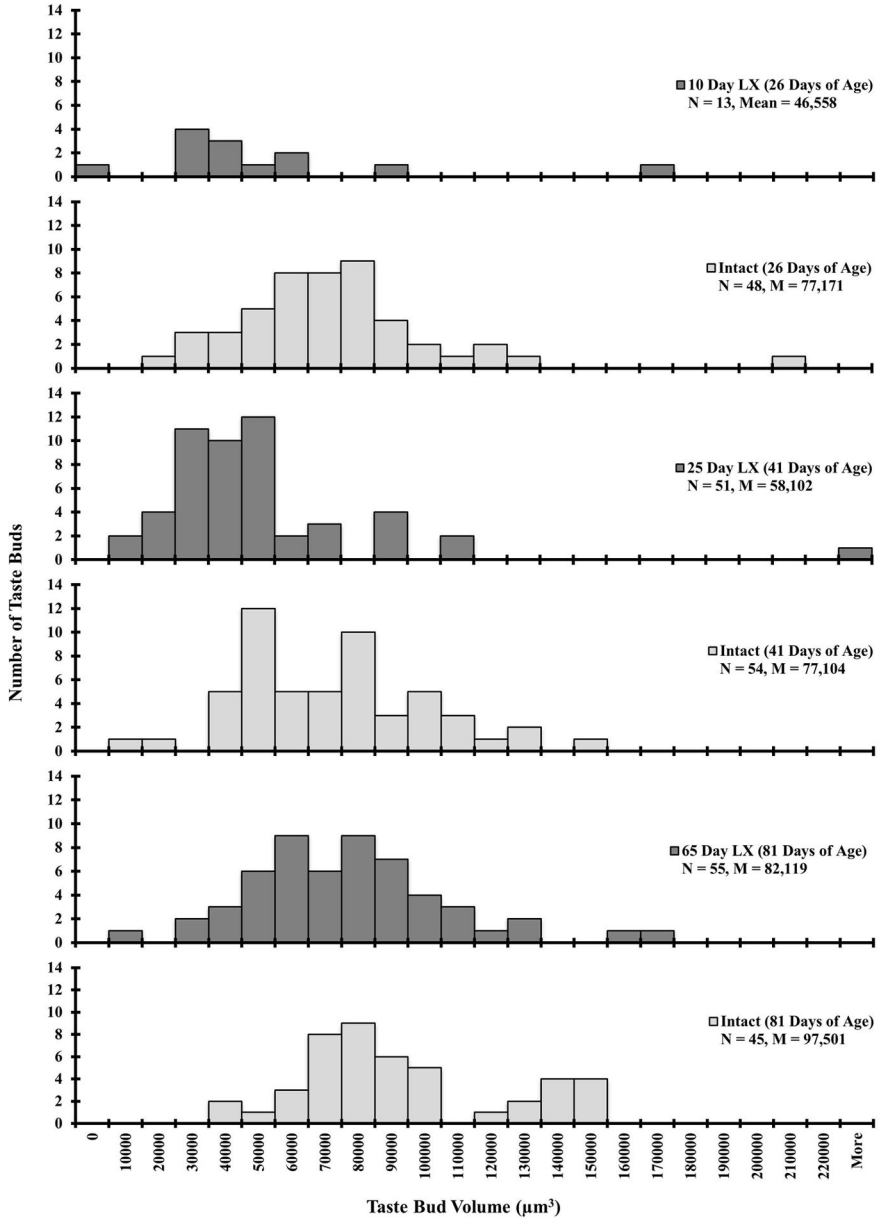

Figure 6.

Taste bud volumes at 16 days after surgery at P10, P25 or P65. Volumes were taken from LX sides and control (intact) sides within the standardized area.

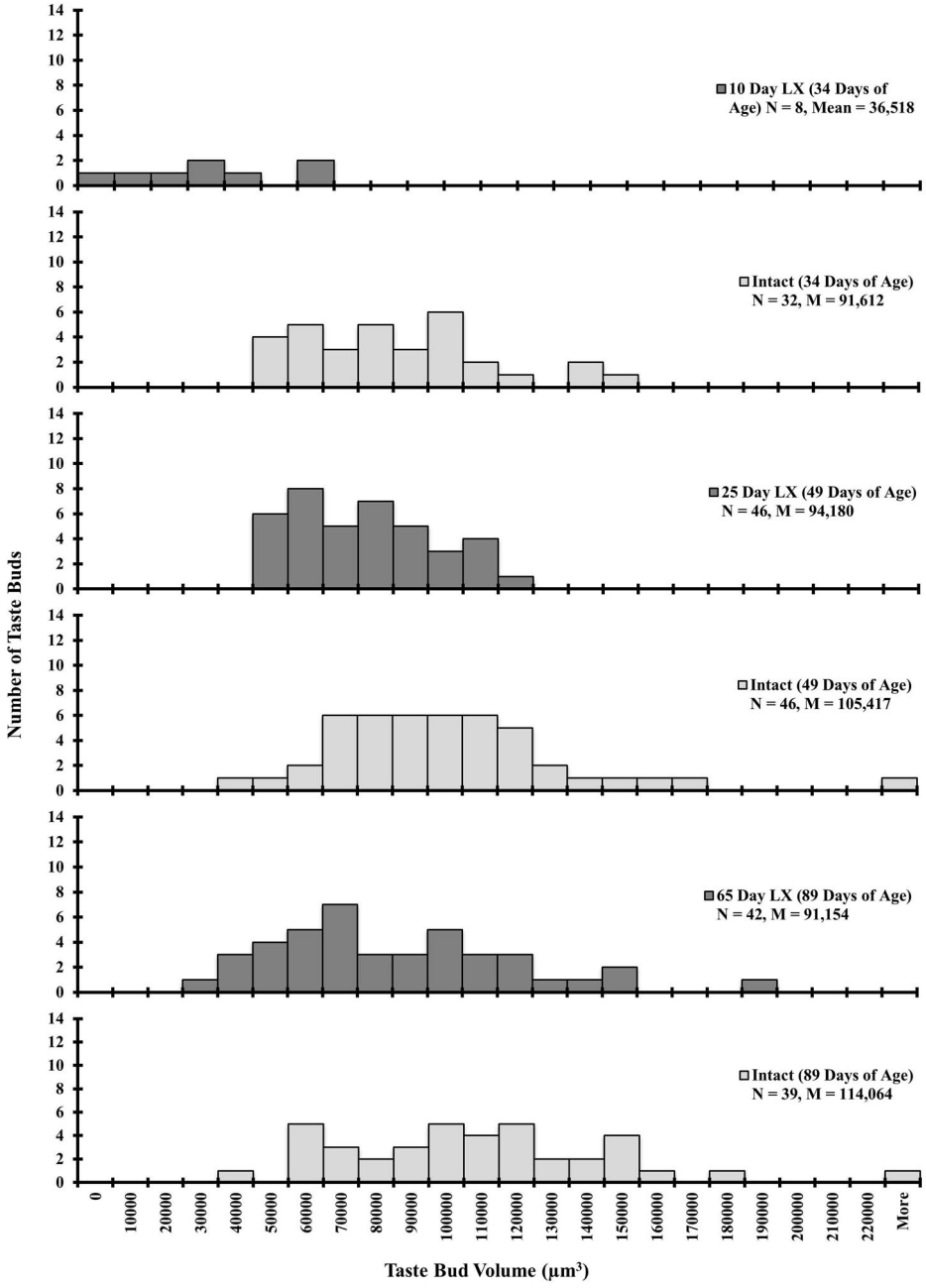

Figure 7.

Tongues were examined 24 days after surgery at 10, 25 or 65 days of age. Volumes from the standardized area were included from both the LX side and control (intact) side of the tongues.

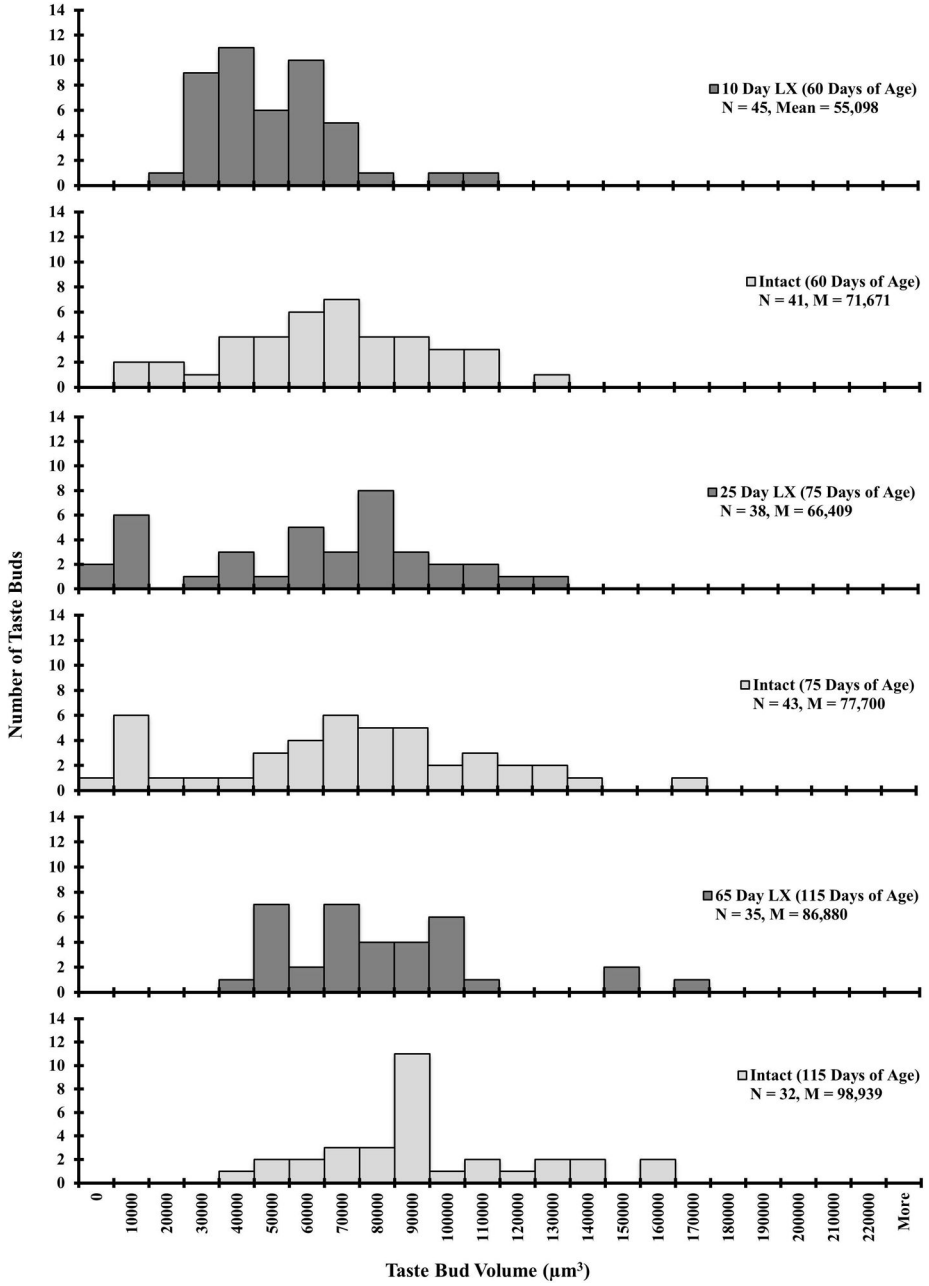

Figure 8.

Rats had LX surgery at 10, 25 or 65 days with taste bud volumes assessed 50 days after surgery on both the LX side and control (intact) side of the tongues. Volumes were obtained across the standardized area of the tongue.

Table 1

Summary of average frequency (± SEM) and percentages of total papillae for each morphological type.

Time Post	Surgical Cond.	No Pore			Pore			Filiform-like		
		P10	P25	P65	P10	P25	P65	P10	P25	P65
2 days post surgery	LX	10.2 ** ± 2.3 (*14.5*)	7.0 ± 1.8 (*10.8*)	6.0 ± 1.5 (*9.2*)	58.0 ± 3.8 (*82.4*)	57.0 ± 4.5 (*89.2*)	58.6 ± 1.1 (*90.8*)	2.2 * ± 1.1 (*3.1*) †	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)
	Intact	3.6 ± 2.7 (*5.7*)	2.2 ± 0.7 (*3.4*)	1.8 ± 1.1 (*2.5*)	66.2 ± 5.3 (*94.3*)	65.4 ± 4.1 (*96.6*)	69.6 ± 2.4 (*97.5*)	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)
8 days post surgery	LX	11.6 * ± 2.3 (*20.3*) †	13.4 ** ± 1.5 (*18.1*) †	7.2 ± 1.7 (*11.1*)	48.0 ± 8.4 (*75.0*) †	60.2 ± 3.3 (*80.6*) †	58.8 ± 4.4 (*87.2*)	2.4 ± 1.5 (*4.7*)	1.0 ± 0.5 (*1.3*)	1.2 ± 0.5 (*1.7*)
	Intact	4.0 ± 1.4 (*5.5*)	2.2 ± 1.2 (*2.8*)	2.8 ± 1.6 (*3.3*)	69.6 ± 5.1 (*94.5*)	74.8 ± 2.5 (*97.2*)	77.6 ± 3.6 (*96.4*)	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)	0.2 ± 0.2 (*0.3*)
16 days post surgery	LX	25.5 ** ± 3.5 (*32.6*) ‡	11.4 ± 2.3 (*14.9*)	9.2 ± 3.5 (*11.5*)	42.4 ** ± 11.5 (*53.6*) ‡	56.2 * ± 3.2 (*78.1*) †	63 ± 4.0 (*81.9*)	10 ** ± 4.3 (*13.8*) ‡	5.6 ± 1.6 (*7.0*)	5.0 ± 1.0 (*6.6*)
	Intact	1.5 ± 0.5 (*1.4*)	1.4 ± 0.5 (*1.7*)	1.2 ± 0.7 (*1.5*)	82.8 ± 5.5 (*98.6*)	80.2 ± 3.0 (*98.3*)	80.2 ± 4.1 (*98.5*)	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)	0.0 ± 0.0 (*0.0*)
50 days post surgery	LX	1.75 ± 0.9 (*2.0*)	3.2 ± 1.4 (*4.4*)	10.0 ± 3.6 (*17.6*)	80.3 ± 5.6 (*98.0*)	66.4 ± 3.9 (*92.7*)	53.6 ± 8.7 (*82.4*)	0.0 ± 0.0 (*0.0*)	2.0 ± 2.0 (*2.9*)	0.0 ± 0.0 (*0.0*)
	Intact	0.3 ± 0.3 (*0.3*)	1.2 ± 0.7 (*1.3*)	5.0 ± 2.5 (*6.5*)	81 ± 5.6 (*99.7*)	77.8 ± 4.4 (*98.4*)	68.4 ± 2.6 (*93.2*)	0.0 ± 0.0 (*0.0*)	0.2 ± 0.2 (*0.3*)	0.2 ± 0.2 (*0.3*)

Transected versus intact sides of the tongue are compared at each surgical age x days post-surgery condition.

Percentages of each papillae type based on the total number of papillae within each surgical condition are listed in parentheses.

For mean counts: *denotes $p < 0.05$

**denotes $p < 0.001$

For percentages: †denotes $p < 0.05$

‡denotes $p < 0.001$