Taste Neurons Have Multiple Inductive Roles in Mammalian Gustatory Development[a]

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ABSTRACT: The embryonic loss of brain-derived neurotropic factor (BDNF)-dependent taste axons in *bdnf* null mutant mice secondarily impairs the development of gustatory epithelia and taste buds. In normal mice gustatory development continues for at least two weeks postnatally as axons promote taste bud formation. We conclude that taste axons in the fungiform, foliate, vallate and nasopalate papillae: i) promote papilla development, and ii) establish competent gustatory cells and iii) mature taste buds. Hence, gustatory innervation contributes critically to at least three of the multiple inductive interactions controlling the development of mammalian gustatory structures.

INTRODUCTION

Perhaps a dozen laboratories are using embryonic and postnatal surgery, organ culture, null mutant mice, transgenic mice, and gene product localization to investigate developing gustatory systems (Ref. 32 and this volume). Questions have been raised recently about the role of innervation in taste bud development.[2,11,28] Immunocytochemical, behavioral, and quantitative morphological assessments of brain-derived neurotropic factor *(bdnf)* null mutant (–/–) mice address some of these concerns.[6,30,41]

ANALYSIS OF GUSTATORY DEVELOPMENT IN BDNF NULL MUTANT MICE

In $bdnf^{-/-}$ mice[7] the prenatal development of taste papillae and taste buds was impaired at all four sites examined: fungiform, foliate, vallate and nasopalate.[6,27,30,41] Neonatal $bdnf^{-/-}$ mice have sparsely innervated vallate papillae, which are poorly developed, and lack most taste buds.[27,41] These three phenotypic features of $bdnf^{-/-}$ tongues are evident in FIGURE 1. Quantitative assessments revealed that both the area of the vallate gustatory epithelium and the number of residual taste buds were linear functions of innervation density.[30] Consequently, the density of BDNF-dependent innervation explains about 90% of the variance in vallate trench wall area and taste bud abundance.

Fungiform taste buds took on varied forms in neonatal $bdnf^{-/-}$ mice. Many fungiform taste buds had ample numbers of appropriately oriented fusiform cells[11] (FIG. 2A–C). Either the abundant lingual nerve somatosensory axons or a few residual chorda tympani taste axons could have contributed to the development of these apparently normal fungiform taste buds (FIG. 3A). However, the majority of $bdnf^{-/-}$ fungiform taste buds were small and displayed a continuum of defects including miss-

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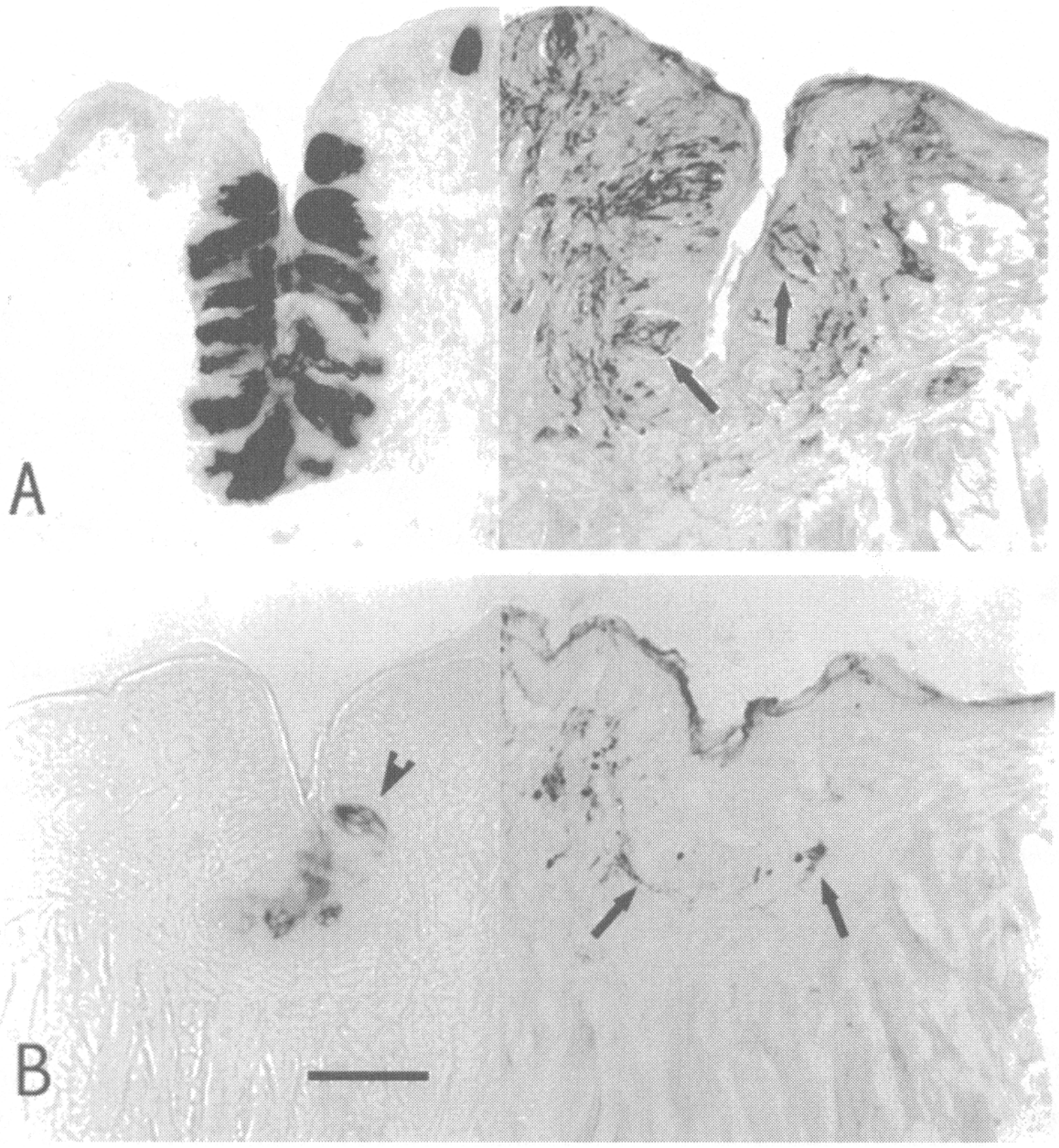


FIGURE 1. Composite photomicrographs have been arranged to display taste buds and innervation in 20-μm cross sections of the vallate papilla in postnatal day 7 (P7) mice. The *left half* is stained for keratin 8 by mAb Troma I and the ABC procedure while the right half is stained for axons by antiserum against PGP 9.5.[19,41] **(A)** *Arrows* indicate clusters of axon terminations in *bdnf* wild-type taste buds. Deep trenches are evident. **(B)** This section contains one of the two vallate taste buds in this *bdnf* null mutant mouse *(arrowhead)*. *On the right* it is evident that the innervation also is sparse *(arrows)* and the trench shallow. *Scale bar* in (B) is 100 μm for all.

ing or misoriented intragemmal cells (FIG. 2D). Complete serial sections showed some fungiform taste buds contained only one or two elongated or immature polygonal taste cells (FIG. 2E,F). *bdnf*$^{-/-}$ vallate taste buds were also smaller on average.[30] These size reductions of sparsely innervated taste buds are consistent with Krimm and Hill's observations on normal rats that the size of a fungiform taste bud is proportional to the number of geniculate neurons that innervate it.[20] *Bdnf*$^{-/-}$ mice lacked several posterior and lateral fungiform taste buds and papillae. Such fungiform papillae developed ectopic filiform spines, presumably reflecting the loss of BDNF-sustained gustatory innervation.[27,30] Similar changes occur after postnatal gustatory denervation.[12,14,25,34,36]

In spite of heavy neuronal losses in *bdnf*$^{-/-}$ mice, variable numbers of neonatal taste neurons were able to survive. Lacking BDNF, these surviving taste neurons appear to

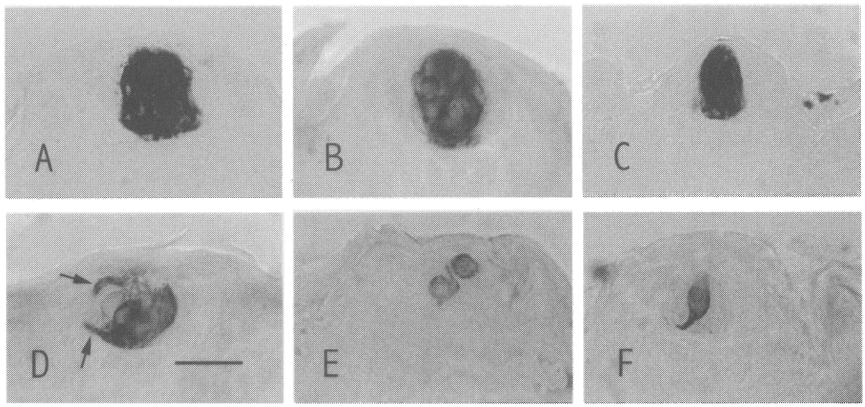
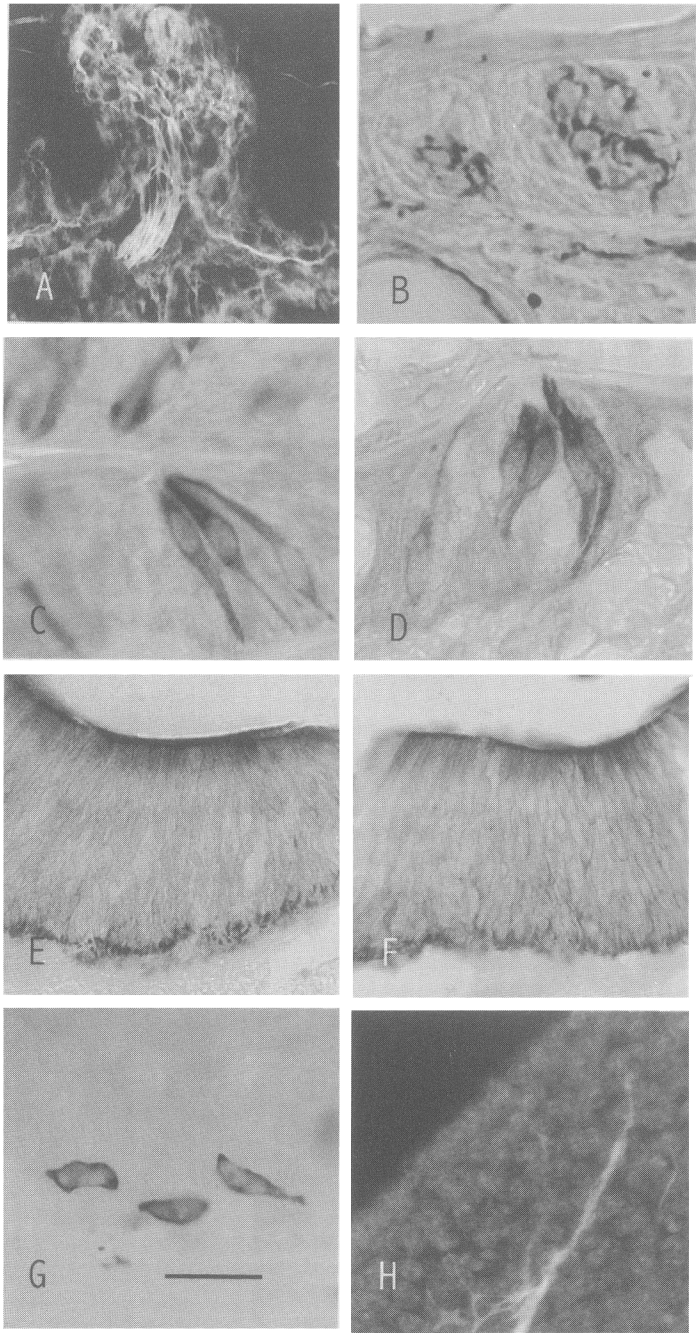


FIGURE 2. Troma I staining of fungiform taste buds in P12 **(A)** *bdnf*$^{+/+}$ and **(B–F)** *bdnf*$^{-/-}$ mouse tongue. *Arrows* indicate misoriented cells in (D). The entire taste bud is visible in (E) and (F). *Scale bar* in (D) is 20 μm for all.

have been supported by functionally redundant trophic factors present in the peripheral targets.[6] Neurotrophin-3 (NT3) is the most likely candidate for a stochastically acquired, taste neuron rescue factor, since NT3 and BDNF are the TrkB receptor ligands normally present in gustatory papillae.[28,29] Genetic breeding experiments should be able to evaluate the capacity of NT3 to act as a functionally redundant neurotrophin that may substitute for BDNF.

We took four approaches in assessing the functionality and differentiation of residual taste receptor cells in *bdnf*$^{-/-}$ mice. First, we determined that *bdnf*$^{-/-}$ taste cells express taste-specific keratin (FIGS. 1B and 2). Second, we immunostained for the G protein gustducin as a taste cell-specific molecule associated with sugar stimulus transduction. Gustducin staining was comparable in *bdnf*$^{+/+}$ and *bdnf*$^{-/-}$ vallate intragemmal cells (FIG. 3C,D). Third, we found that residual vallate taste buds were innervated. Innervation was evident both with double-stained taste buds (monoclonal antibodies (mAbs) Troma I and RMO-270) and in richer detail with protein gene product (PGP) 9.5 immunostaining of intragemmal nerve fibers within ovoid taste buds that could be visualized by Nomarski optics (FIG. 3B). Fourth, behavioral testing provided a simple but telling test of functionality. Some residual taste buds must have been functional because postnatal day 12 (P12) *bdnf*$^{-/-}$ and P12 *bdnf*$^{+/+}$ mice were indistinguishable in their significant selective ingestion of 0.3 M sucrose over distilled water. Behavioral tests of preferential ingestion had to be carried out with minimal stress, for we found, as have others, that selective responses to taste solutions failed to occur when *bdnf*$^{-/-}$ mice were physically restrained.[27,30] Selective sucrose ingestion requires: innervated taste receptor cells, an effective hedonic code for 'sweet,' and functional afferent connections with the brain.

Evidence is lacking for BDNF support of nongustatory neurons in the oral and nasal mucosa. An initial survey of the *bdnf*$^{-/-}$ vomeronasal mucosa found no gross structural changes (FIG. 3E,F). Merkel cells continued to survive in P12 *bdnf*$^{-/-}$ mice (FIG. 3G), whereas substantial numbers of neonatal Merkel cells die in neurotrophin-3 null mutant (*nt3*$^{-/-}$) mice.[1] Both our group and Nosrat, Olson and colleagues noted that many lingual somatosensory neurons were absent in *nt3*$^{-/-}$ mice.[27,30,41]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INDUCTIVE STEPS IN GUSTATORY MORPHOGENESIS

The term 'induction' is employed in nearly 10,000 biomedical abstracts per year. Its use is spurred by the abundance of fate-determining interactions that generate state changes in gene expression, cell function, tissue organization, and organ composition. Developmental processes employ cascades of unidirectional and bidirectional inductive events. An induced tissue (*e.g.,* the neural tube) may be transformed into an inducer (the floor plate), which is able in turn to induce adjacent cells (into motor neurons[37]). Throughout an organism's development there are multiple inductive events within and between the three embryonic germ layers. Since "virtually every vertebrate tissue and organ is formed by some type of induction,"[18] gustatory development is unlikely to be exempt from manifold cellular interactions.

All instances of embryonic induction require the presence of a tissue that produces the inductive signal and another tissue that responds to it. *Competence* is the capacity of a target to respond to an *inductive* influence that *determines* a cell's fate. A tissue's competence to respond to an inductive stimulus is time dependent. After losing competence to respond to one inducer, a maturing embryonic tissue may gain responsiveness to another. Cells generally acquire the competence to respond to inductive chemicals either by countering an inhibitory factor or by synthesizing a missing signal transduction component, such as a receptor. For example, fibroblast growth factor (FGF) induces sympathetic neurons to synthesize the receptor TrkA thereby making the neurons competent to respond to nerve growth factor (NGF) release.[4]

Although it complicates matters, assessments of gustatory development ought to consider specification of the papilla's spatial location and the morphogenetic emergence of the four principal structural components: gustatory innervation, receptor cells clustered within taste buds, basal cells whose daughters replace aged taste receptor cells, and in most instances a gustatory papilla having additional morphological specializations such as trenches and rich somatosensory innervation. The development of taste papillae and taste buds is surely controlled by a cascade of multiple inductive events. The mammalian gustatory gene cascade may involve the segmentation gene sonic hedgehog and its putative receptor complex, patched/smoothened, as well as bone morphogenetic protein 4, and a distal-less homologue among others.[5,13,24]

Our results with *bdnf* null mutant mice reveal that innervation is crucial for the prenatal morphogenesis of the vallate papilla and taste buds. Even if papilla placement and the initial steps in papilla formation occur before innervation,[10,22,39] the profound defects of the $bdnf^{-/-}$ vallate papilla indicate that, at the least, innervation bolsters papilla development. Axons may act on epithelial cells directly, or indirectly by actions mediated by innervated mesenchymal tissue (FIG. 3H). There has been disappointingly little characterization of the actions of gustatory axons between the time they first arrive at the vallate (E11–12) and the birth of the mouse more than a week later. It is probable that nascent taste buds are under construction during much of the last prenatal week, for

FIGURE 3. **(A)** In fungiform papillae of $bdnf^{-/-}$ mice, lingual nerve fibers form a rich plexus (mAb RMO-270 against neurofilaments, gift of V. Lee). **(B)** Intragemmal axonal varicosities are evident in a small and a large P7 $bdnf^{-/-}$ taste bud. (Polyclonal antiserum PGP9.5 1:1200 dilution, with the ABC method.[41]) Individual taste receptor cells were immunopositive for polyclonal antiserum against gustducin (Santa Cruz Biotechnology) in P7 **(C)** $bdnf^{+/+}$ and **(D)** $bdnf^{-/-}$ vallate taste buds. The vomeronasal epithelia of **(E)** $bdnf^{+/+}$ and **(F)** $bdnf^{-/-}$ P7 mice were comparably immunoreactive for keratin 8. **(G)** Keratin 8-immunopositive Merkel cells were present in $bdnf^{-/-}$ mouse nasopalate. **(H)** Nerve fibers approach the dorsal lingual epithelium in E10.5 wild-type mice; mAb RMO-270. *Scale bar* in (G) is 20 μm for (A–D,G,H) and 45 μm for (E,F).

in neonatal rats it requires about ten days for vallate taste buds to mature.[35] We found that *bdnf* null mutation disrupted the development of those few vallate taste buds that normally mature *pre*natally.[6,30,41] Embryonic denervation produced by a neurotoxin provides independent preliminary evidence for the prenatal nerve dependency of gustatory papillae and taste buds. Morris-Wiman *et al.*[23] injected the neurotoxin β-bungarotoxin into E12 mouse embryos. At E18 the lingual epithelium lacked the lingual sensory axons and the fungiform papillae and taste buds that were present in sham-injected controls.

One might anticipate lineage restrictions of multipotent gustatory progenitor cells during the last prenatal week. But a critical cell determination step that irreversibly establishes vallate gustatory competence has been shown to be predominantly under *post*natal neural control in rat.[33] Taste bud maturation is also a predominantly *post*natal event in rat.[15,35] Mice are similar in that the great majority of their vallate taste buds mature postnatally.[6] Neural control of postnatal taste bud development is required to explain the greater than 4-fold increase in the numbers of postnatal vallate taste buds that arise in the presence of two IXth nerves rather than one.[16,17] Hence, developmental experiments indicate that innervation contributes prenatally to taste papilla formation, while postnatally it establishes the gustatory competence of the epithelium and triggers taste cell differentiation. Denervation at P1 caused the disappearance of rat fungiform taste buds and the conversion of fungiform papillae into filiform papillae.[25] As rats mature, the nerve dependence of fungiform taste buds is progressively reduced.[3,12,14,25,26,34,38,39] With this exception, innervation is essential for both the adult maintenance of mammalian taste buds as well as their perinatal development.[32,33,37]

CONCLUSIONS

The embryonic loss of taste neurons with *bdnf* null mutation results in stunted gustatory papillae and a shortfall of taste buds. Since a solitary differentiated taste cell can exist in a fungiform papilla, it seems that receptor cells need not aggregate into a multicellular bud to survive. Sense organ nerve dependence is not restricted to the gustatory system. Perinatal denervation impairs the development of mechanoreceptors such as Pacinian corpuscles, Meissner corpuscles, muscle spindles, and Golgi tendon organs.[40] Similarly, *nt3* null mutant mice suffer a profound loss of proprioceptive neurons and secondarily fail to develop muscle spindles and Golgi tendon organs.[8,9,21] It was to be anticipated from the postnatal nerve dependence of fungiform papillae and taste buds[25] that the embryonic loss of BDNF-dependent mouse taste neurons would also impair the development of gustatory papillae and taste buds. It is probable that taste axons contribute sequentially to papilla formation, gustatory competence, and taste bud formation. Even a fixed neural signal can lead to sequential responses if cellular competence changes with time. The only exception to the *post*natal nerve dependence of mammalian taste buds are some fungiform taste buds that postnatally relax their nerve dependence. Our studies of null mutation-induced *pre*natal losses of taste neurons demonstrate that embryonic gustatory development is nerve dependent. The molecular assessment of regulatory gene cascades will provide additional opportunities to investigate mechanisms of gustatory development.

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