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Lingual deficits in BDNF and NT3 mutant mice leading to gustatory and somatosensory disturbances, respectively

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SUMMARY

A combination of anatomical, histological and physiological data from wild-type and null-mutated mice have established crucial roles for BDNF and NT3 in gustatory and somatosensory innervation of the tongue, and indeed for proper development of the papillary surface of the tongue. BDNF is expressed in taste buds, NT3 in many surrounding epithelial structures. Absence of BDNF in mice leads to severely malformed taste bud-bearing papillae and severe reduction of taste buds, a loss of proper innervation of remaining taste buds and a loss of taste discrimination although not of the suckling reflex *per se*. In contrast, absence of NT3 leads to a massive loss of somatosensory innervation of lingual structures. These findings demonstrate distinct roles for BDNF and NT3 in the establishment of the complex innervation apparatus of the tongue with non-overlapping roles for the lingual gustatory and somatosensory systems. The distinction between different sensory modalities, being dependent on either BDNF or NT3 may also have clinical implications.

Key words: taste buds, sensory organ, gustatation, neurotrophins, knockout, lingual

INTRODUCTION

Taste buds are chemosensory organs consisting of collections of spindle-shaped modified epithelial cells that transduce chemical stimuli into neural signals. Large numbers of taste buds reside in three taste areas of the tongue; fungiform, foliate and circumvallate papillae. The lingual epithelium also contains numerous non-gustatory, filiform papillae which may also serve as tactile endorgans (Nosrat et al., 1996). Although lingual taste buds receive specific gustatory innervation from the 7th and 9th cranial nerves, the surrounding epithelium, as well as the filiform papillae receive an extensive somatosensory type of innervation from cranial nerves V and IX. While the taste buds and the taste receptor cells within them are innervated by gustatory nerves, conveying mainly gustatory stimuli, many fibers conveying general somatosensory stimuli are found immediately surrounding the taste buds and in the lingual epithelium. In a gustatory papilla, those fibers that enter taste buds at their base and have synaptic contacts with taste cells are denoted intragemmal fibers while nerve fibers found outside the taste bud proper are denoted perigemmal fibers. In addition, some perigemmal fibers enter the taste buds without synapsing on taste cells (Nagy et al., 1982; Finger et al., 1990; Yamasaki et al., 1984).

It is assumed that taste buds in mammals are induced in local epithelium by ingrowing nerves (Vintschgau and Hönigschmied, 1876; Torrey, 1940; Farbman and Mbiene, 1991). Recent studies indicate that taste buds indeed form from local epithelium (Barlow and Northcutt, 1995; Stone et al., 1995). However, in urodele, taste buds develop in the absence of proper innervation (Stone, 1940; Wright, 1964; Barlow et al., 1996). The capacity for differentiation into taste buds resides in certain epithelia and not in others (Zalewski, 1974) and indicates that lingual epithelium is specialized for taste buds before nerves arrive. In addition, we have also shown that the gustatory, but not the surrounding epithelium of the developing rat tongue expresses brain-derived neurotrophic factor (BDNF) which therefore probably selectively supports the gustatory innervation (Nosrat and Olson, 1995). Cell turnover studies in mature taste buds (Beidler and Smallman, 1965; Farbman, 1980), indicate that renewal and maturation of taste cells, as well as formation of synaptic contacts, are continuous processes also in the adult. Low levels of neurotrophin expression may thus be needed in taste buds throughout life. In rats, BDNF and neurotrophin 3 (NT3) mRNAs are expressed in specific patterns in the lingual epithelium suggesting gustatory and somatosensory functions, respectively (Nosrat et al., 1996).

The neurotrophins BDNF and NT3 (see Barde, 1989; Johnson and Oppenheim, 1994; Barbacid, 1995) play roles in the innervation of sensory organs, such as the inner ear (Ernfors et al., 1995), vibrissae (Ibanez et al., 1993), carotid body (Hertzberg et al., 1994; Erickson et al., 1996) and muscle spindles (Ernfors et al., 1994b). They are expressed in the target areas that are going to become innervated. Neuronal loss has been observed in sensory but not motor neurons in mice

carrying null-mutations for these neurotrophins (BDNF$^{-/-}$: Ernfors et al., 1994a; Jones et al., 1994, NT3$^{-/-}$: Ernfors et al., 1994b; Farinas et al., 1994). Nerve cell bodies providing sensory innervation to the tongue lie in different cranial ganglia. The gustatory ganglia including the geniculate (n. VII), petrosal (n. IX) and nodose (n. X) ganglia, and cranial ganglia related to general sensory innervation of the tongue, including the trigeminal (n. V) and petrosal (n. IX) ganglia, all show neuronal losses in BDNF and NT3 null-mutated mice.

To elucidate the roles of BDNF and NT3 in the tongue, we examined mRNA expression patterns in mouse tongues. Structures and innervation patterns of the tongue were then studied in wild-type and BDNF and NT3 null-mutated mice. The gustatory papillae and taste buds were malformed in BDNF knockouts. To examine possible gustatory disturbancies in these mice, two different behavioral tests were applied. BDNF knockouts showed behavioral deficiencies indicating severe impairment of taste discrimination ability. NT3 null-mutated animals displayed severe loss of somatosensory innervation.

MATERIALS AND METHODS

In situ hybridization

Adult Balb-C mice (n=4, B&K Universal, Sollentuna, Sweden) were killed by cervical dislocation and the tongues dissected and frozen with liquid CO_2. Sagittal and transverse sections (14 μm) were cut on a cryostat and thaw-mounted onto coated slides (ProbeOn, Fisher). Two non-overlapping oligonucleotide probes complementary to mouse *BDNF* (50-mer probes from bases 123 and 569, GenBank accession number X55573; Hofer et al., 1990) and mouse *NT3* (50-mer probes from bases 409 and 851, GenBank accession number X53257; Hohn et al., 1990) were synthesized (DNA Technology, Denmark) and used for in situ hybridization (Dagerlind et al., 1992; Nosrat et al., 1996). These oligonucleotide probes had no similarities to other sequences deposited in GenBank and each pair generated identical in situ hybridization patterns.

Immunohistochemistry

Homozygous mice were bred from breeding stocks of mice with targeted disruptions of the BDNF or NT3 genes (Ernfors et al., 1994a,b) by mating heterozygous animals. BDNF (postnatal day 15 (P15) n=5, P16 n=2, P21 n=2, P24 n=2) and NT3 (P1 n=2, P12 n=2 and P16 n=1) null-mutated mice and wild-type controls were used. Animals were genotyped by polymerase chain reaction, as previously described (Ernfors et al., 1995). Antibodies against protein gene product 9.5 (PGP 9.5, Biogenesis Ltd., Great Britain) were used (diluted 1:400) to maximize visualization of the innervation apparatus of the tongue. Antibodies to PGP 9.5 seem to be the most inclusive neuronal marker yet for thin peripheral fibers (Wilson et al., 1988). Procedures for immunohistochemistry were according to Hökfelt et al. (1973). Sections were analyzed using epifluorescence microscopy (Nikon Microphot-FXA). Selected areas were also documented using confocal microscopy (BIO-RAD MRC-600).

Scanning electron microscopy

Three P14 BDNF$^{-/-}$, three P12 NT3$^{-/-}$ and three control mice were perfused with either Karnovsky's solution (Karnovsky, 1965) or the fixative used for immunohistochemistry. Tongues were dissected out and postfixed for 2 hours in the same solutions, rinsed in PBS, dehydrated in a graded series of ethanol, using acetone and tetramethyl silane as final steps. They were then mounted on aluminium stubs, platinum coated with the sputter technique and examined in a scanning electron microscope (Joel JSM 820) (see Blomlöf and Lindskog, 1995). For quantitative analysis, all fungiform papillae were counted at a magnification of 500×. Individual papilla morphology and presence of a taste pore was determined at a magnification of 1000-1500× by two observers. For statistical evaluations of the quantitative analysis of fungiform papillae, Anova with Fisher PLSD test was used.

Independent ingestion test

The test procedure was in accordance with that of Hall and Bryan (1980). Pups (16-day old, 2 BDNF knockouts and 5 controls from the same dam) were transferred to heated (32±1°C) animal cages and deprived of maternal care as well as opportunity to suckle and get milk for 3 hours. Just before testing, pups were removed from the deprivation incubator, and stimulated to empty their bladders and defecate by stroking the anogenital region with a soft artist's brush for about 30 seconds. They were then weighed to the nearest 10^{-3} g. Knockout animals weighed much less than wild-type litter mates. For testing, pups were placed for 30 minutes in round glass containers (7 cm in diameter), inside a test incubator (37°C). The entire floors of the test containers were covered with cloth towels which had been generously soaked with 0.5 M sucrose solution. The surface of the towels was just barely covered with the solution. In this way, the pups were exposed to a thin but uniform layer of sugar solution. The pup behavior was observed by two observers. After testing, animals were dried and again weighed.

Taste discrimination test

Pups were transferred to heated (32±1°C) animal cages and deprived of maternal care as well as opportunity to suckle and get milk. The deprivation period was 1 hour for the first test and 1.5 hours for the following tests. P16 pups (2 BDNF knockouts and 5 controls from the same dam) were taken out from their heated cages after the deprivation period, one at a time and, holding them by the shoulders and using a Pasteur pipette, they were allowed to first suckle on a 1 M sucrose solution (or the solution was placed on their mouths) and thereafter on a 1 M NaCl solution. Reactions to the solutions were studied by two independent observers. To expand the taste discrimination test, two new groups of pups (one knockout and six controls from one dam, one knockout and five controls from another, P16 and 17) first received 1 M sucrose solution, then 1 M NaCl and then quinine hydrochloride (Q-HCl) solutions (0.01 M and 0.1 M after each other). The pups were then returned to their cages and placed with their parents for 1.5-2 hours. The tests were then repeated with the same pups. This time the order of presentation of the four different test solutions was varied and after each presentation of a salt or Q-HCl solution an additional test with sugar solution was included. A typical test of this kind would thus include 7 solutions, e.g. sugar, salt, sugar, 0.01 M Q-HCl, sugar, 0.1 M Q-HCl, sugar. This procedure allowed observation of ingestion behavior to a preferable taste after having been offered tastes that were disliked. The following day, the test was repeated twice, with varying orders of offering the bitter, salt and sugar solutions. Two additional new groups of pups (P12 and P19, 1 knockout and 3 controls in each group) were also tested. The test procedure, timing and order of giving the taste solutions was similar. After the final test, the animals were genotyped.

RESULTS

BDNF and NT3 mRNA in mice

BDNF mRNA labeling was located over taste buds of circumvallate (Fig. 1A,B), foliate and fungiform papillae (Fig. 1H). In circumvallate papillae, the labeling was found in basal parts of taste buds and in a fraction of cells located more towards the taste pore (Fig. 1B). NT3 mRNA labeling was found in some epithelial cells of filiform papillae (Fig. 1J) and lingual

epithelium, and in deeper layers of the superior and lateral epithelium of fungiform papillae (around the taste bud proper, Fig. 1I). On the superior surface of the circumvallate papilla and in its vicinity, we observed delineated, plate-like areas where the epithelium was thinner (and the lamina propria thicker) than in its surroundings. We call these structures circumvallate plates (CP). NT3 mRNA, but not BDNF mRNA, labeling was abundant in the epithelial cells of CPs (Fig. 1C,D,F,G). Labeling was low in the deeper layers of the circumvallate trench epithelium as well as in basal layers of the outer surface and crypt epithelium of the foliate papillae. Expression of BDNF and NT3 mRNAs in mice is therefore related, in a non-overlapping fashion, to gustatory and somatosensory structures of the tongue, respectively. The neurotrophin mRNA expression patterns in mice matches those seen in rats (Nosrat et al., 1996).

Immunohistochemistry

Based on the restricted expression of BDNF and NT3 in the mouse tongue, we assumed that they would play distinct roles in gustatory and somatosensory innervation of the tongue. To examine this possibility, and to maximize detection of nerve fibers, irrespective of their neurotransmitter or neuropeptide content, antibodies to protein gene product 9.5 (PGP) were used. PGP immunoreactivity is also observed in some taste cells of multiple taste bud-bearing lingual papillae (Wakisaka et al., 1996; and our own observations). The morphology and innervation of taste buds and lingual papillae of wild-type, BDNF$^{-/-}$ and NT3$^{-/-}$ mice are summarized in Table 1.

Innervation of the tongue in wild-type animals

The tongue of control mice was richly innervated by PGP-IR nerves. A subepithelial nerve plexus was seen on both ventral and dorsal surfaces, although the plexus on the dorsal surface contained more fibers (Fig. 2A). Filiform papillae carried a variable amount of intraepithelial fibers (Fig. 2A,B). Already at P1, fungiform papillae were richly innervated (Fig. 2C), and their taste buds received many intragemmal nerve fibers resembling the innervation pattern in adults (Fig. 2C,D). A PGP-positive taste cell was occasionally noted in fungiform papillae taste buds in adults. Some fibers terminated very close to the taste pore. The rest of the fungiform papilla was also richly innervated by varicose fibers with specific termination patterns (Fig. 2D). Circumvallate papillae were also richly innervated, and many PGP-positive cells were encountered in each individual taste bud (Figs 2E, 3A). A thick subepithelial nerve plexus, surrounding the papilla, was seen (Fig. 3A). In CPs (Fig. 2F), a specific pattern of ramifying nerve fibers was seen. A thick nerve bundle ran in the lamina propria just underneath the epithelium (Figs 2F, 3B). Fibers then entered the epithelium, ramified perpendicular to the surface, and came very close to the outer surface. Most of the fibers terminated almost at the same level, exhibiting varicosities along the way and a

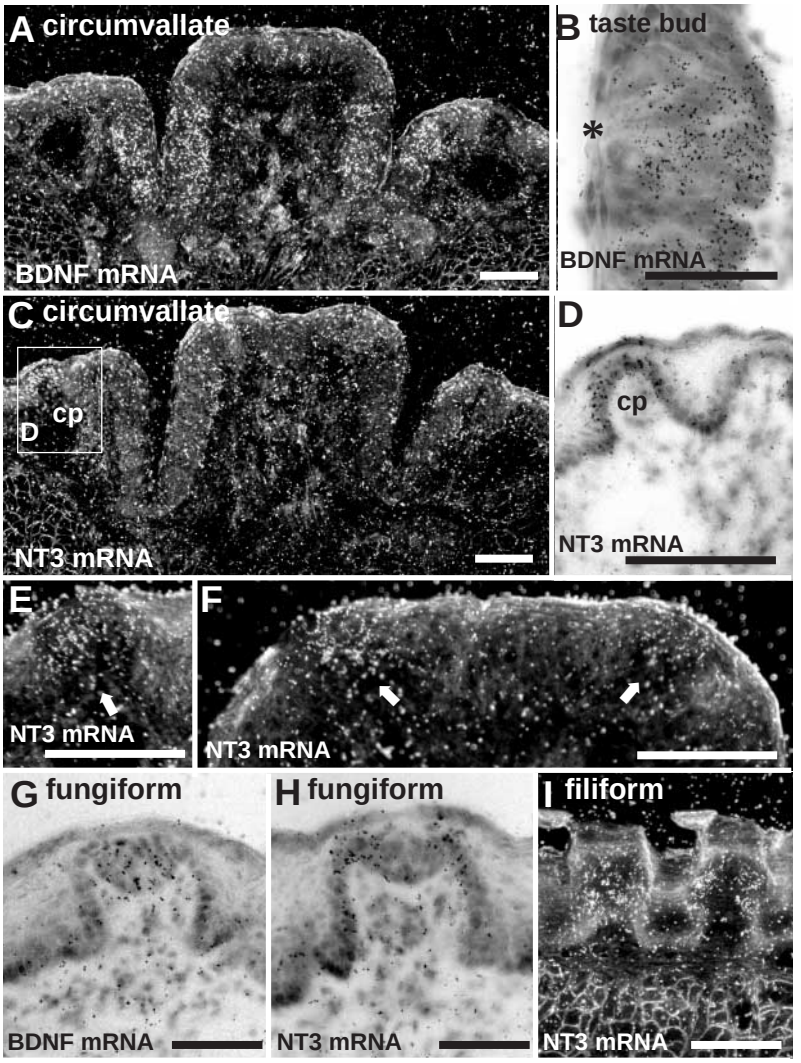

Fig. 1. Sections through different adult mouse tongue structures, labeled by in situ hybridization with mouse BDNF and NT3-specific probes. Scale bars represent 100 μm in A and C-F, and 50 μm in B and G-I. Sections were photographed under dark- or bright-field illumination. (A) Transverse section of a circumvallate papilla. BDNF mRNA labeling is observed above taste buds, both in the trench epithelium and on the superior surface epithelium. (B) Higher magnification of a circumvallate taste bud. BDNF mRNA labeling is found above cells located basally and in a fraction of cells located more towards the taste pore (asterisk). (C) High density of NT3 mRNA labeling is found in basal epithelial cells of circumvallate plates (CP). (D) Higher magnification of CP (boxed area) in C. At CPs, the epithelium is thinner (and the lamina propria is thicker) than its surroundings. (E) Another example of NT3 mRNA labeling in a transversally sectioned CP (arrow) in another animal. (F) CPs on the middle top surface of circumvallate papillae contain NT3 mRNA labeling (arrows). (G) Sagittal section of a single taste bud-bearing fungiform papilla. BDNF mRNA labeling is observed above the taste bud. (H) NT3 mRNA labeling is mainly found in perigemmal epithelial cells, surrounding the taste bud proper, and in deeper layers of the lateral epithelium of the fungiform papilla. (I) Transverse section of two posterior filiform papillae. NT3 mRNA labeling is found in deeper layers of the epithelium in the lateral walls of these non-gustatory papillae.

Table 1. Comparison of morphological and histological findings in wild type, BDNF$^{-/-}$ and NT3$^{-/-}$ mice

Genotype	Papillae	Characteristic findings
Wild type+/+	Filiform papillae and lingual epithelium	Well innervated and well developed, intraepithelial nerve fibers and subepithelial nerve plexus
	Fungiform papillae	Varicose intra- and perigemmal nerve fibers, well developed taste buds carrying taste pores
	Circumvallate papillae	Large number of taste buds, extensive subepithelial nerve plexus, CP* well innervated
BDNF$^{-/-}$	Filiform papillae and lingual epithelium	Filiform papillae not affected, reduction of fibers in the subepithelial nerve plexus
	Fungiform papillae	35% decrease in number, partial loss of intragemmal fibers, perigemmal nerve fiber population unaffected, morphologically atypical papillae and papillae with no taste pore were observed among the remaining papillae
	Circumvallate papillae	Malformed, reduction in size, reduction in number of taste buds, disorganized taste buds, reduction of subepithelial nerve fibers corresponding to the loss of taste buds, CP innervation unchanged
NT3$^{-/-}$	Filiform papillae and lingual epithelium	Total loss of intraepithelial innervation, hypodeveloped filiform papillae
	Fungiform papillae	Number unchanged, flat superior surface, taste buds well innervated, few perigemmal nerve fibers in close proximity of taste buds
	Circumvallate papillae	Large number of taste buds, taste buds well innervated, large number of fibers subepithelially, malformed CP, CP not innervated

*CP, Circumvallate plates

larger varicosity at their termination. Taste buds were also observed at the top surface, mainly in the superior surface epithelium of the papilla, between the two trenches. Foliate papillae were richly innervated, with many PGP-positive fibers and cells in the foliate papillae taste buds. The positive cells, as in the circumvallate papillae, were spindle shaped.

Innervation of the tongue and papillae morphology in BDNF$^{-/-}$ mice

The filiform papillae and lingual epithelium were well innervated and fibers were found in the subepithelial plexus. The innervation pattern of filiform papillae resembled that of wild-type animals (Fig. 2G,H). Many fungiform papillae had an atypical morphology, and were lacking a recognizable taste pore (see Fig. 5). Papillae with normal morphology and taste buds, but with fewer intragemmal fibers were also found (Fig. 2G,I). The perigemmal innervation apparatus was unchanged. The distribution of fungiform papillae was restricted mainly to the anterior part of the tongue (in wild-type and NT3 knockouts, the fungiform papillae are also found further posteriorly). Circumvallate papillae were reduced in size and contained far fewer and less disturbed taste buds compared to controls (Figs 2L,M,O-Q and 3C). There was a striking reduction in the amount of nerve fibers surrounding the circumvallate papillae (Fig. 3C), corresponding to the loss of taste buds. A similar reduction in the amount of nerve fibers was observed in all gustatory papillae. The amount of intragemmal fibers was also decreased in the few remaining taste buds. Fewer taste buds were found in foliate papillae.

As the animals became older (over P20) fewer nerve fibers were generally observed and the fibers that remained showed a decreased PGP-IR fluorescence intensity. Many fungiform papillae on the anterior part of the tongue contained few, if any intragemmal nerve fibers in their taste buds (Fig. 2J). Papillae, morphologically resembling large atypical filiform papillae, were observed in the anterior part of the tongue in the older mice (Fig. 2K). Such papillae contained large central nerve bundles in their connective tissue core, giving off many thinner branches that entered and traversed the epithelium. These papillae were akin to atrophic 'filiform-like' fungiform papillae, found following nerve transections (Oakley et al., 1990; Nagato et al., 1995). The morphologically distinct intraepithelial nerve plexus of CPs resembled that of wild-type animals and the fiber density had not decreased (Figs 2M,O,Q and 3C). Circumvallate papillae taste cells were further deranged, and taste bud organization was further distorted in the older mice (Fig. 2P,Q).

Innervation of the tongue and papillae morphology in NT3$^{-/-}$ mice

At P1, there was much less innervation of the tongue in NT3$^{-/-}$ compared to wild-type animals and the subepithelial nerve plexus was virtually absent. We did not observe any nerve fibers in the filiform papillae. Nerve fibers were observed in the connective tissue core of the fungiform papillae, going to the middle part of the superior surface where the taste bud is located (Fig. 2T). These nerve fibers were fewer than in normal animals. Circumvallate and foliate papillae were still developing in P1 mice. The nerve plexa (surrounding these papillae) were clearly visible and some PGP-positive taste cells observed in the developing papillae. The superior surface taste buds of circumvallate papillae were more developed than the taste buds in the trenches.

At P16, filiform papillae remained non-innervated (Fig. 2R,S). Taste buds of the fungiform papillae were well innervated (Fig. 2R,U) but few perigemmal fibers were seen in close vicinity to the taste bud proper. The circumvallate papilla taste buds were well innervated and had normal morphology (Fig. 2V,W). Large numbers of nerve fibers were seen surrounding the papillae. Notably, CPs were lacking innervation (arrow in Fig. 2V).

Tongue surface morphology

The gross morphology of the papillae appeared abnormal in microscopical sections of BDNF and NT3 mutant mice. In order to better evaluate tongue surface appearance and papillae morphology, we examined tongues of wild-type and mutant mice using scanning electron microscopy.

SEMs of filiform, fungiform and circumvallate papillae were examined in 14-day old BDNF knockout mice.

Filiform papillae appeared less mature than in wild-type animals, in terms of length, keratinization and appearance of the spine, especially at the intermolar eminence (IME; Fig. 4F,I compared to 4A,D).

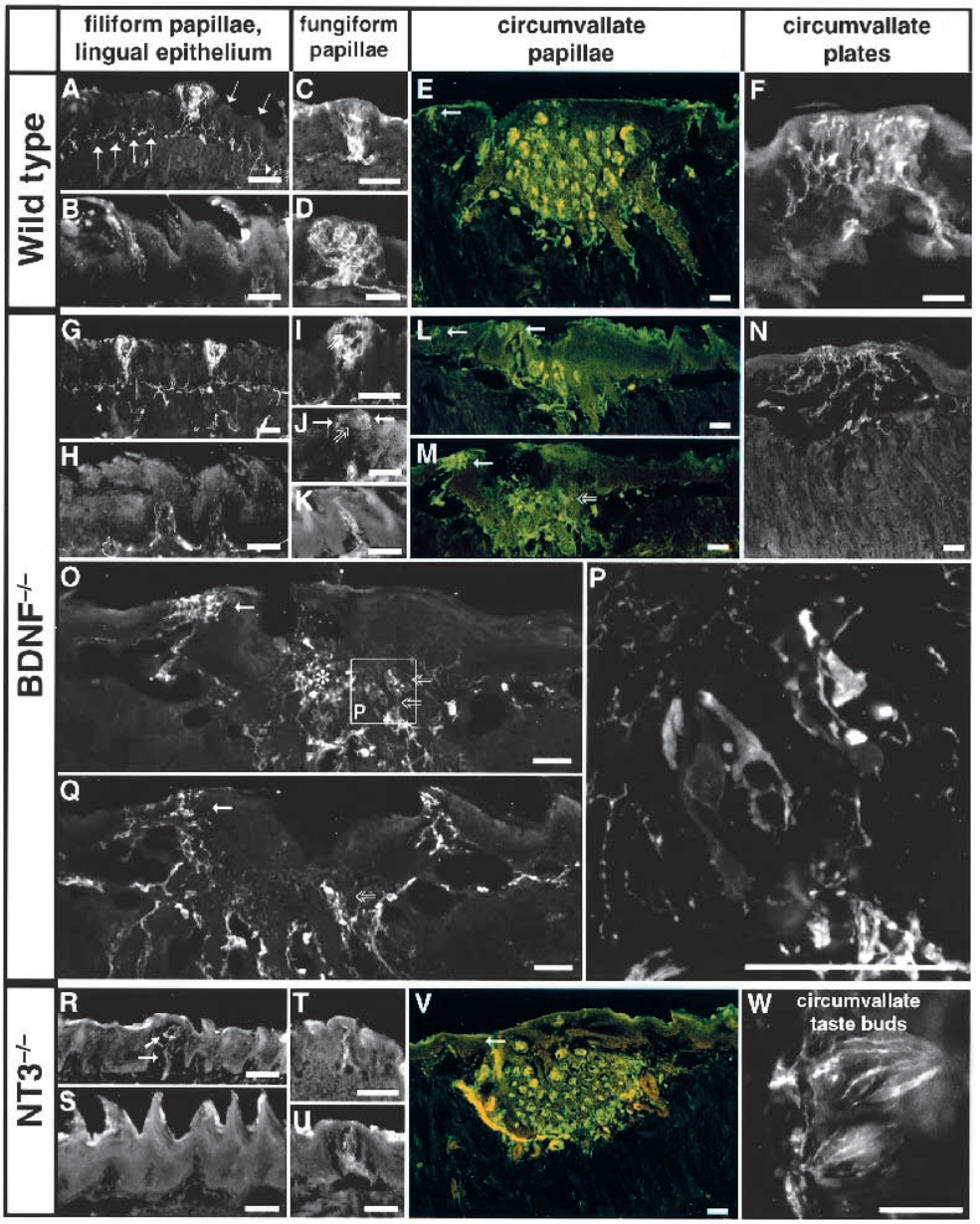

Fig. 2. Protein gene product 9.5 (PGP) immunoreactivity in different parts of the tongue of wild-type, BDNF$^{-/-}$ and NT3$^{-/-}$ mice. Scale bars represents 50 μm. Circumvallate papillae are sectioned at the same levels (medial trench wall), Q is sectioned at the lateral trench wall. (A) Anterior part of the tongue in P15 wild-type mouse, showing the subepithelial nerve plexus, intraepithelial nerve fibers in anterior filiform papillae (arrows), and innervation pattern in a fungiform papilla. (B) Intraepithelial fibers are also seen in the posterior filiform papillae. (C) A fungiform papilla at P1. The papilla is well innervated. Both intra- and perigemmal innervation patterns can be distinguished at this young age. (D) A fungiform papilla (P15) in the anterior part of the tongue. The papilla is innervated by varicose nerve fibers, both within the taste bud proper (intragemmal innervation) and in the surrounding epithelium (perigemmal innervation). (E) A P15 circumvallate papilla is richly innervated and many taste cells are PGP-positive. Many taste buds are seen as rounded fluorescent structures. Due to higher fluorescence intensity, positive taste cells appear yellow. Nerve fibers appear green. The green color of the outermost layer of the epithelium is due to non specific fluorescence. The intraepithelial fibers seen posterior to the papilla (arrow) belong to a circumvallate plate (CP). (F) A sagittally sectioned CP. At CPs, a nerve bundle gives off many intraepithelial fibers with varicosities along their passage in the epithelium and terminate with swellings (cf. figure 3B). (G) Anterior part of the tongue in a P15 BDNF$^{-/-}$ mouse, showing the subepithelial nerve plexus, intraepithelial nerve fibers in anterior filiform papillae, and innervation pattern in two fungiform papillae. Note that there is a lesser amount of intragemmal fibers in the taste buds than in normal animals while the perigemmal innervation is well developed. (H) Intraepithelial fibers are also found in the posterior filiform papillae. (I) There is a lesser amount of intragemmal nerve fibers in the taste buds (arrow) of BDNF$^{-/-}$ mouse fungiform papillae. (J) There is further reduction of the intragemmal nerve fibers of P21 animals (delineated arrow) but the perigemmal innervation pattern remains (filled arrows). (K) A well innervated, large filiform-like papilla in the anterior part of the tongue of a P21 BDNF knockout mouse (the anterior filiform papillae are smaller and do not contain as many nerve fibers, compared to two neighboring filiform papillae located anterior and posterior, one on each side). These papillae probably represent atrophic fungiform papillae. (L) Circumvallate papilla of a P15 BDNF knockout mouse. It has a different morphology and fewer taste buds compared to wild-type and NT3 knockouts. Arrows are pointing at CPs. (M) Circumvallate papilla at P21. There is disorganization in the taste buds and taste cells are less PGP-positive and do not have normal morphology (delineated arrow, see also O, P and Q). Note that CP is still well innervated (filled arrow). (N) Sagittal section through several CPs. The dark areas underneath the CPs are large cavernous blood vessels found in normal animals as well (cf. Fig. 3A). (O) Confocal micrograph of M at higher magnification. Only two taste buds (delineated arrows) are observed in the medial trench wall. There is also an area with large amount of nerve fibers (asterisk). Boxed area represents figure P. (P) Higher magnification confocal micrograph of the taste buds in O. These taste buds appear disorganized. (Q) Confocal micrograph of a section through the lateral trench wall of P21 BDNF$^{-/-}$ mouse. Only one taste bud (delineated arrow) is observed. Solid arrow points at innervation in a CP. (R) Anterior part of the tongue in a P16 NT3 knockout mouse. There is virtually total loss of the subepithelial nerve plexus and intraepithelial nerve fibers in the anterior filiform papillae and the perigemmal innervation apparatus of the fungiform papillae. The taste bud of the fungiform papilla is innervated (arrows). (S) No intraepithelial fibers are seen in the posterior filiform papillae. (T) A nerve bundle in the center part of a P1 fungiform papilla going towards the middle part of the superior surface of the papilla, where the developing taste bud is located (compare Fig. 2C). (U) A P16 fungiform papilla. Nerve fibers are found only intragemmally. (V) A P16 circumvallate papilla containing many well innervated taste buds and PGP-positive taste cells. Taste buds are seen as rounded fluorescent structures. No CP-like innervation pattern (in the area of the filled arrow) or intra-epithelial nerve fibers are observed in the superior surface epithelium (there is a taste bud on the superior surface). (W) Higher magnification of two circumvallate taste buds. Taste cells have normal morphology and are PGP-positive. Intragemmal nerve fibers are also PGP-positive.

The number of fungiform papillae was decreased by 35% in BDNF knockouts (see Fig. 5) and the remaining papillae were of smaller size than in wild-type or NT3 mutant mice (Fig. 4F compared to A,K). Many remaining fungiform papillae did not have a visible taste pore (see Fig. 5). Many atypical fungiform papillae were also observed (Figs 4G,Q-S and 5). In all of the studied animals only two atypical and no other fungiform papillae were observed posterior to the IME (fungiform papillae were observed further posterior to IME in normal and NT3 knockouts, Fig. 4L).

The trench system of circumvallate papillae in mice consists of two crescent-like trenches (Fig. 4E), in contrast to one 'U' shaped trench system in rats. The rounded plate-like structures on the superior surface of the papillae, and surrounding the trench are the CPs (Fig. 4E). Some CPs seemed to contain a taste bud, since taste pores appeared to be present on the superior surface. Circumvallate papillae in BDNF knockouts showed a distorted morphology. There was markedly increased keratinization on the superior surface. CPs were however well developed. The papillae were of smaller size in BDNF knockouts than in wild-type and NT3$^{-/-}$ mice (Fig. 4J, compared to E and O) and a more 'V' shaped shallow trench system was seen.

In NT3 knockout animals, the tongue surface did not look as mature as in wild-type animals, especially not at the IME (Fig. 4K,N). The gustatory papillae had the same gross appearance as in wild-type animals but had a smoother surface. The number of fungiform papillae was unchanged and the superior surface of the papillae had a visible taste pore (Figs 4M, 5). Fungiform papillae were observed posterior to IME (Fig. 4L) as in wild-type animals. The trench system of the circumvallate papillae had the same organization as in normal animals, but CPs were absent (Fig. 4O).

Independent ingestion

Preweanling rat and mouse pups discriminate between taste qualities. For instance, they prefer sweet solutions to water, as shown by the weight increase differences noted after ingestion of either solution (Hall and Bryan, 1981). The higher the sugar concentration, the more weight increase. Observing the pups during the test period would also give further information about their ingestive behavior. We carried out the following test to investigate if weight increase after the test compared to initial body weight would suggest different consumption rates in BDNF$^{-/-}$ and control mice.

Both wild-type and heterozygous pups became calm quickly after they were put in the test containers. They licked their paws and occasionally we noticed that a pup had a drop of the sugar solution between both paws in front of it and showed licking behavior. The BDNF null-mutated animals were hyperactive for a long period of time and did not show any ingestive behavior. They rolled on the floor of the test container and became less active only at the last minutes of the test period, most probably because of exhaustion. The wild-type and heterozygous pups gained weight during the test period (0.231-0.483 g) while the knockouts did not (0.008-0.018 g).

Taste discrimination

The independent ingestion test indicated clear differences in ingestion behavior between knockouts and controls. However, since BDNF mutant animals were hyperactive during the procedure and since their balance is disturbed, their lack of ingestion of the sugar solution might be related to non-gustatory disturbances such as proprioceptive difficulties. Therefore, we also carried out the taste discrimination test, delivering various taste solutions directly to the mouth.

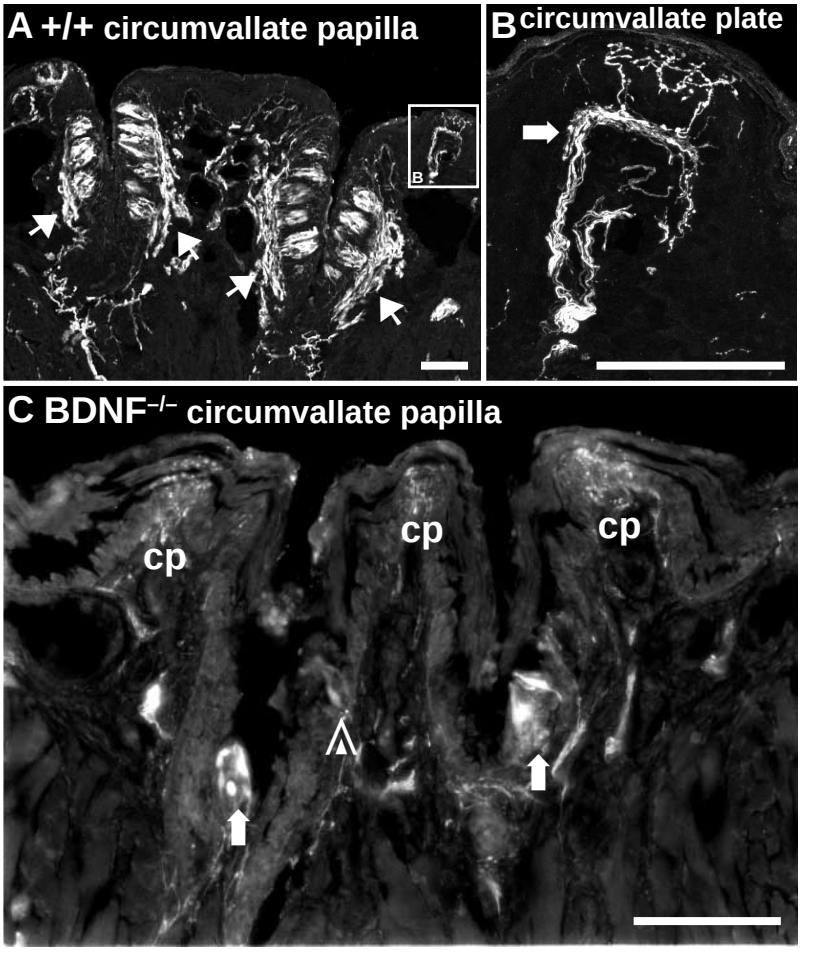

Fig. 3. (A) Confocal micrograph of a transversally sectioned adult circumvallate papilla. Scale bar represents 100 μm. The subepithelial nerve plexus is visible. Taste buds contain PGP-positive taste cells and nerve fibers. Circumvallate plates (CP) are seen on each side. The CP on the left side contains a taste bud. Large cavernous blood vessels are also observed in the area of the circumvallate papilla. (B) Higher magnification of a CP (boxed area in A). Scale bar represents 100 μm. A thick nerve bundle is seen in the superior parts of the dermal papilla, underneath the epithelium (arrow). Varicose nerve fibers traverse the epithelium and end in close proximity to the outer surface epithelium. (C) A transversally sectioned circumvallate papilla of a BDNF$^{-/-}$ mouse at P16, corresponding to A. Scale bar represents 100 μm. Only 3 taste buds are observed (arrows). The delineated arrow is pointing at an atypical taste bud. CPs are well-innervated. Note that the subepithelial nerve plexus (compare arrows in A) is diminished and contains only a few fibers, corresponding to the reduction in taste bud number.

The wild-type and heterozygous pups both drank the sugar solution. They showed tongue movements (mouthing) through the whole sugar solution ingestion period, and appeared to prefer the sweet taste. Many pups would also taste and drink a small amount of the 1 M salt solution but then refused to drink more of it. They showed strong aversion to bitter taste (refusing, rejecting, spinning of the tail and movements). Wild-type and BDNF$^{+/-}$ mice would often drink sugar solution again after the other solutions (salt and bitter). When a test series started with salt followed by sugar and bitter solutions, or

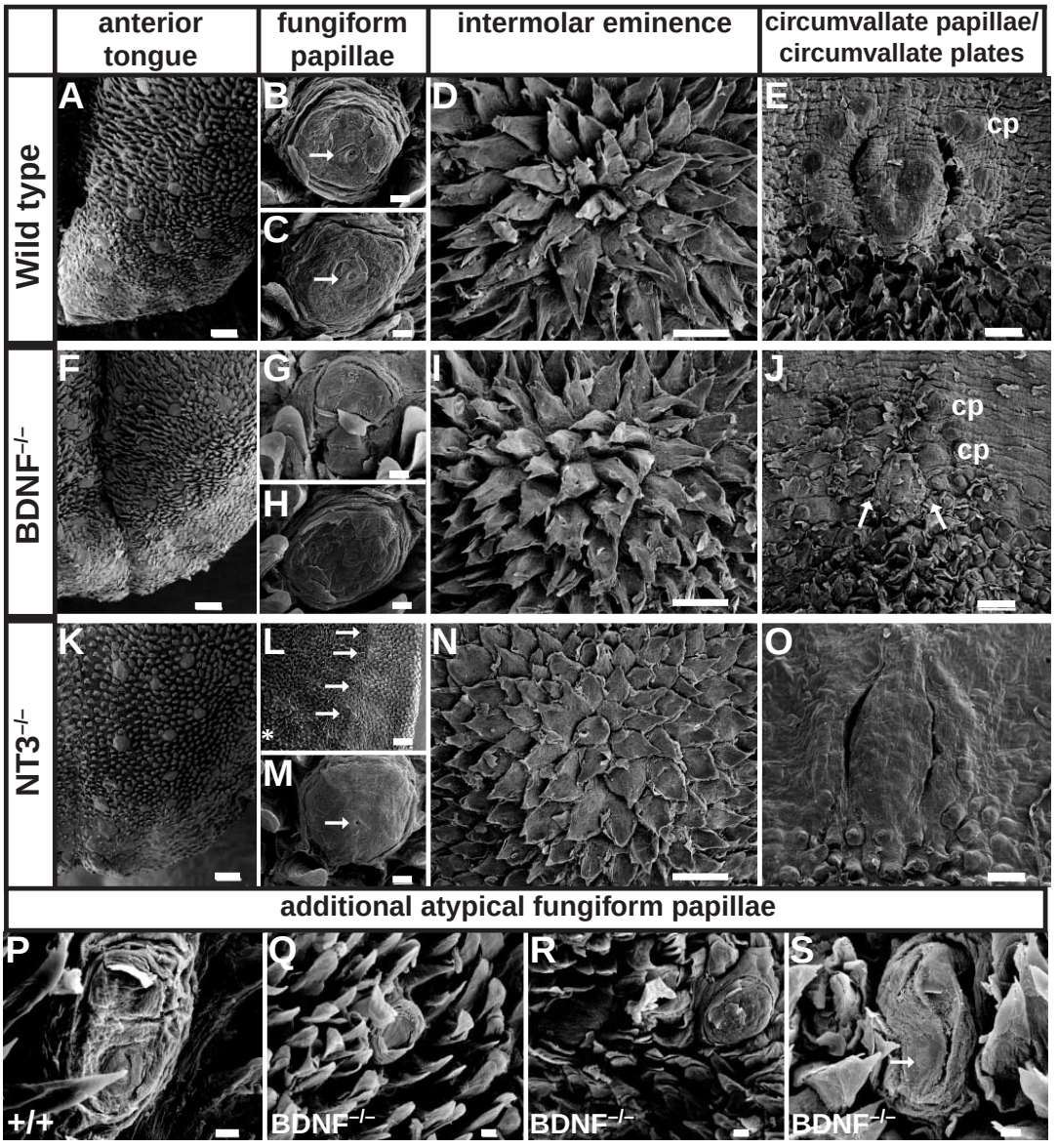

Fig. 4. Scanning electron micrographs of mice tongues. Scale bars represent 10 μm in B,C,G,H,M,P-S, and 100 μm in A,E,F,J,K,L,O, and 150 μm in D,I and N. Anterior is down in all figures. (A) Anterior part of a tongue in a wild-type mouse (postnatal day 14 = P14). Fungiform papillae are rising above the filiform papillae and contain a taste pore on the superior surface. (B,C) Two examples of anterior fungiform papillae with taste pores (arrows) on the superior surface. (D) Intermolar eminence (IME). Filiform papillae in this region appear to lie in concentric layers around a center. Filiform papillae are coarse and well-keratinized and the IME has a mature appearance. (E) A circumvallate papilla and its two crescent-like trenches on each side. Circumvallate plates (cp) are the round, large structures located in the periphery and on the superior surface of the papillary part between the trenches. (F) Anterior part of the tongue in a BDNF knockout. Fungiform papillae in BDNF knockouts appear smaller than in normal (and NT3 knockout) mice and they do not rise above the anterior filiform papillae. (G) An atypical anterior fungiform papilla, having a filiform-like spine at its periphery. (H) A fungiform papilla with normal appearance, which does not contain a taste pore. (I) At the IME, filiform papillae are smaller. (J) A circumvallate papilla with a distorted morphology. It is smaller than in normal and NT3 knockout mice. More loose surface keratinization is observed and the trench system is 'V' shaped and shallow. Arrows indicate location and direction of the V-shaped trench. Circumvallate plates (cp) are easily observable. (K) Anterior part of the tongue in NT3 knockout mouse (P12). Many fungiform papillae are seen and they rise above the filiform papillae and tongue surface. (L) Fungiform papillae (arrows) are observed posterior to IME (asterisk) and have a normal appearance (and also have a visible taste pore). (M) A fungiform papilla with a well-observable taste pore. The superior surface has a smoother appearance than in wild-type and BDNF knock-out animals. (N) Filiform papillae at IME are smaller and show less keratinization. (O) The gross appearance of the circumvallate papilla in NT3 knockouts resembles that of normal animals. Note that circumvallate plates are not well-developed in these animals and that the surface has a smoother appearance than in wild-type and BDNF knockout animals. (P-S) Examples of atypical fungiform papillae. (P) A twin-fungiform papilla found in a wild-type animal. These papillae are located posterolaterally to IME. (Q) A small fungiform papilla partly covered by an overgrowing epithelial sheath in a BDNF$^{-/-}$ mouse. This papilla did contain a pore on its superior surface (not visible on the micrograph). (R) Two fungiform papillae in a BDNF$^{-/-}$ mouse. The fungiform papilla located to the left is flat, and has a spine on its periphery. The superior surface of the fungiform papilla to the right is partly covered by desquamated epithelium. (S) A large and atypical fungiform papilla in a BDNF$^{-/-}$ mouse that does contain a visible taste pore (arrow).

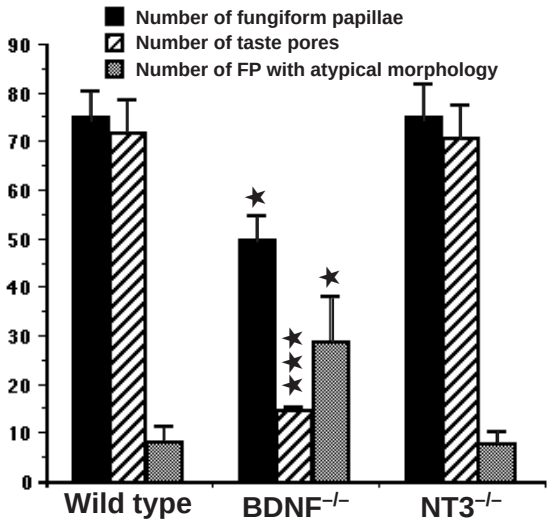

Fig. 5. Quantitative analysis of scanning electron microscopy data on fungiform papillae (FP). Mean counts ± s.e.m. are given. BDNF null-mutated mice have significantly fewer papillae, the remaining FP have fewer visible taste pores and a large number of the remaining FP have atypical morphology compared to both wild-type and NT3 null-mutated mice. Significances compared to both wild type and $NT3^{-/-}$ are: ★ $P<0.05$; ★★★ $P<0.001$, ANOVA with Fisher's PLSD test.

bitter, salt and sweet solutions, they showed clear preference for the sweet taste.

The BDNF knockout animals did show some suckling reflex. Thus when the first drop of any of the test solutions was placed on their mouth, they displayed some initial jaw movements and drank some of the solution but stopped after a short period of time. This behavior was more distinct at the beginning of the tests. The knockouts did not show any reactions indicating preference for the sugar solution, nor aversion to bitter taste, or tasting and refusing to drink more of the salt solution as seen in control animals. The animals were generally calm at the beginning of the tests, but occasionally became anxious, attempting to bite the pipette or the examiner's finger, and when held, became hyperactive. Aggression and refusing to taste the solutions were also observed in a few +/+ and +/– pups (mainly in older groups). For an overview of the results see Table 2.

It has been shown that rat pups show robust preference for sucrose and aversion to quinine by 15 days of age, behaviors that are also seen in adult animals (Hall and Bryan, 1981).

Table 2. Summary of behavioral responses of wild type, $BDNF^{+/-}$ and $BDNF^{-/-}$ mice in taste discrimination test

	Test solutions		
Animals	Sweet (1 M)	Salt (1 M)	Bitter (0.1 and 0.01 M)
Control animals*	+++ (n=22, ×9)	(+)– (n=22, ×9)	– – – (n=17, ×8)
$BDNF^{-/-}$	(+) (n=6, ×9)	(+) (n=6, ×9)	(+) (n=4, ×8)

*Pooled $^{+/+}$ and $^{+/-}$ animals.

+++ preference, (+)– dislike, – – –aversion, (+) transitional suckling reflex with no taste preference or aversion. Numbers of animals followed by the number of times that the test was repeated are given within parentheses.

NaCl solution delivered to P15 rats by infusion through cannulae to different parts of the mouth (anterior, middle and posterior in relation to IME) are treated as aversive (Kehoe and Blass, 1985), especially when the cannulae were in anterior and middle positions. Our results from control animals are in agreement with these reports. We therefore conclude that taste discrimination ability is impaired in BDNF mutant mice.

DISCUSSION

Conventional gene knock-outs allow studies of the consequences of development in the absence of a given protein. Our data strongly demonstrate the importance of BDNF for proper structural and functional development of the gustatory apparatus and hence for taste discrimination. Similarly, our data demonstrate that NT3 is crucial for proper development of the complex somatosensory innervation of the tongue.

Anatomical, histological and functional (in BDNF mutant mice) disturbances observed in the tongues of BDNF and NT3 knockout animals match the neuronal deficits in the cranial ganglia of such animals. The neuronal loss in BDNF knockout mice reported in the petrosal (and nodose) ganglion (Conover et al., 1995; Jones et al., 1994) might be partly due to the loss of gustatory neurons due to the loss of BDNF expression in the gustatory target tissue. Taste buds in the anterior part of the tongue are innervated by the chorda tympani nerve. About 48% of the geniculate ganglion neurons contribute fibers to chorda tympani and the greater petrosal nerves (Gomez, 1978). There is a possibility that the 50% loss of neurons of the geniculate ganglion in BDNF knockouts that does not change with time (counted at E18.5, P0 and P14), represents loss of the gustatory neurons (Liu et al., 1995). In rats, BDNF mRNA is expressed in the gustatory epithelium in different oral regions, including the gustatory epithelium of the fungiform papillae at E15 and thereafter (Nosrat and Olson, 1995). Chorda tympani fibers are seen in the developing fungiform papillae by E16 and enter the gustatory epithelium at the papilla apex by E17 (Farbman and Mbiene, 1991). BDNF mRNA expression in adult taste cells, in both basal cells and cells closer to the taste pore, suggests that basal cells produce BDNF to support the ingrowing nerve fibers and perhaps terminal innervation. BDNF could also then be needed for synaptogenesis due to cell turnover in taste buds. Our finding that BDNF mRNA is also expressed in a fraction of taste cells, in areas where mature taste cells are located, would also suggest that BDNF is required for the maintenance of taste cell innervation.

Null-mutation of the NT3 gene leads also to a 60-70% loss of neurons in the trigeminal ganglion and a 30% loss of neurons in petrosal-nodose ganglia (Ernfors et al., 1994b; Farinas et al., 1994). Loss of NT3 in lingual structures might partly be involved in the loss of neurons in the above-mentioned ganglia. NT3 is a survival factor for sympathetic (DiCicco-Bloom et al., 1993; Birren et al., 1993) and sensory neuron precursor cells (ElShamy and Ernfors, 1996). Like NGF, NT3 can also induce sprouting and terminal branching in target fields (Schnell et al., 1994). Epithelial cells in somatosensory-related oral structures, such as in CP, filiform papillae and superior surface epithelium of the fungiform papillae express high levels of NT3 mRNA, and a very specific axonal branching is observed in these regions. NT3 gene

mutation leads to virtually total loss of intraepithelial nerve fibers in all of the above-mentioned lingual structures.

Although BDNF knockouts were unable to differentiate between taste qualities, we saw some jaw movements and suckling reflexes in these animals when a drop of solution was placed on their mouth, irrespective of the taste quality. NT3 expression appears to be normal in BDNF$^{-/-}$ mice, giving rise to an apparently normal somatosensory cutaneous innervation. This, presumably allows the BDNF$^{-/-}$ mice to feel that solution is placed on their mouth and might trigger the initial jaw movements. In wild-type animals, NT3-dependent perigemmal nerve fibers in the gustatory papillae enter the taste buds but do not synapse on taste cells (Yamasaki et al., 1984). Whether the nerve fibers found in the taste buds of BDNF mutant mice (especially in younger animals) properly synapse on taste cells or not, remains to be elucidated.

The BDNF knockouts have particularly severe malformation of those tongue papillae carrying multiple taste buds, such as foliate and circumvallate papillae, while the single taste bud fungiform papillae appeared less affected initially. The close proximity between BDNF producing sites (taste buds) and NT3 producing sites (epithelial cells surrounding taste buds) in fungiform papillae may explain the differences observed between single taste bud- and multiple taste bud-bearing papillae. However, the proportion of atrophic fungiform papillae appeared to increase postnatally in BDNF$^{-/-}$ animals, suggesting that the presence of NT3 (and/or other trophic factors) could only partly compensate for the lack of BDNF at early stages.

Loss of BDNF affected gustatory papillae morphology. Maintenance of papillae morphology might be exerted directly by nerves but it is also possible that gustatory nerves exert their trophic effect on papillae indirectly through maintenance of functional taste buds. Notably, although initiation of papillae morphogenesis does not require innervation nor nerve induction, maintenance of the already formed papillae requires innervation (Farbman and Mbiene, 1991; Mbiene et al., 1996). This in turn strengthens the hypothesis that proper innervation of taste buds maintains papillae morphology. It is interesting to note that the atrophic fungiform papillae, as well as loss of taste buds in circumvallate papillae noted here in BDNF$^{-/-}$ mice are similar to changes observed previously following nerve transection (Oakley et al., 1990; Nagato et al., 1995). Denervation of fungiform papillae leads to what has been regarded as degeneration of taste buds and filiform appearance of the papillae (Oakley, 1993) although atrophic taste buds have been reported to persist in fungiform papillae in hamster long after nerve transection (Whitehead et al., 1987; Whitehead and Kachele, 1994). It seems that nerves have heterogeneous effects on fungiform papillae, presumably due to the fact that fungiform papillae constitutes a heterogeneous group of papillae.

Morphological changes in the fungiform papillae of NT3 knockouts are not severe. Fungiform papillae with different disturbed morphologies, innervation patterns and functional status (assuming that only fungiform papillae with taste pores have proper gustatory function) are only found in BDNF knockouts. Nerve transection during a sensitive period of circumvallate papillae development leads to loss of the majority of taste buds (Hosley et al., 1987). Similarly, the majority of taste buds are lost in BDNF knockouts. It has been assumed that other sensory nerves might substitute for the gustatory innervation and lead to regeneration of taste buds following nerve transections (see Kinnman and Aldskogius, 1988). Our results suggest that when the taste buds do not produce the proper gustatory neurotrophic factor (BDNF), the trophic function of the nerve on taste bud and papillae maintenance disappears. Indeed, all the atypical forms of fungiform papillae described from nerve transection studies are seen in the BDNF$^{-/-}$ animals.

We also observed a clear difference between fungiform, circumvallate and foliate papillae with regard to PGP-positive cells in their taste buds. This suggests that taste buds in different areas are not homogenous, indicating functional heterogeneity between taste buds at different locations.

The morphological differences noted in the knockouts could be due to different innervation losses, underdevelopment or malnourishment. Since the changes are different in BDNF versus NT3 null-mutated animals we assume that the main cause for malformations is the loss of specific innervation of the tongue. In order to establish if these morphological differences would lead to functional disturbances, we performed taste tests on BDNF knockouts, showing that these animals indeed had functional deficits. NT3 animals could not be maintained long enough to allow testing of gustatory or oral somatosensory functions. The P16 NT3 knockout pup studied was a fortuitous exception, not available for functional tests.

Compensatory mechanisms such as up- or downregulation of other genes must be taken into account when interpreting the net effects of a given null mutation. These concerns notwithstanding, our findings have established distinct roles for BDNF and NT3 in lingual sensory functions. We conclude that BDNF and NT3 are of major importance for proper lingual gustatory and somatosensory innervation, respectively. Gene disruption of these neurotrophins leads to specific anatomical, histological and physiological deficits. The distinction between different sensory modalities, being dependent on either BDNF or NT3, may have clinical implications.

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