Chem. Senses 30: 531–538, 2005

doi:10.1093/chemse/bji046
Advance Access publication July 19, 2005

Expression of Amiloride-sensitive Epithelial Sodium Channels in Mouse Taste Cells after Chorda Tympani Nerve Crush

Noriatsu Shigemura[1], Abu Ahammad S. Islam[1], Chiharu Sadamitsu[1,2], Ryusuke Yoshida[1], Keiko Yasumatsu[1] and Yuzo Ninomiya[1]

[1]Section of Oral Neuroscience, Graduate School of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan and [2]Laboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Correspondence to be sent to: Yuzo Ninomiya, Section of Oral Neuroscience, Graduate School of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. e-mail: nino@dent.kyushu-u.ac.jp

Abstract

Our previous electrophysiological study demonstrated that amiloride-sensitive (AS) and -insensitive (AI) components of NaCl responses recovered differentially after the mouse chorda tympani (CT) was crushed. AI responses reappeared earlier (at 3 weeks after the nerve crush) than did AS ones (at 4 weeks). This and other results suggested that two salt-responsive systems were differentially and independently reformed after nerve crush. To investigate the molecular mechanisms of formation of the salt responsive systems, we examined expression patterns of three subunits (α, β and γ) of the amiloride-sensitive epithelial Na^+ channel (ENaC) in mouse taste cells after CT nerve crush by using *in situ* hybridization (ISH) analysis. The results showed that all three ENaC subunits, as well as α-gustducin, a marker of differentiated taste cells, were expressed in a subset of taste bud cells from an early stage (1–2 weeks) after nerve crush, although these taste buds were smaller and fewer in number than for control mice. At 3 weeks, the mean number of each ENaC subunit and α-gustducin mRNA-positive cells per taste bud reached the control level. Also, the size of taste buds became similar to those of the control mice at this time. Our previous electrophysiological study demonstrated that at 2 weeks no significant response of the nerve to chemical stimuli was observed. Thus ENaC subunits appear to be expressed prior to the reappearance of AI and AS neural responses after CT nerve crush. These results support the view that differentiation of taste cells into AS or AI cells is initiated prior to synapse formation.

Key words: amiloride-sensitive epithelial sodium channel, in situ hybridization, nerve crush, taste bud

Introduction

The chorda tympani (CT) nerve innervating the anterior part of the tongue contains two types of NaCl-responsive fibers. One, the N-type, receives input from receptor cells and the NaCl responses are strongly inhibited by amiloride, a blocker of the epithelial sodium channel (ENaC). In contrast, the E-type fiber receives input from cells poorly sensitive or insensitive to amiloride (Ninomiya and Funakoshi, 1988; Hettinger and Frank, 1990; Ninomiya, 1996, 1998). The glossopharyngeal (IXth) nerve, innervating the posterior tongue, consists primarily of the E type (Formaker and Hill, 1991; Ninomiya *et al.*, 1991; Ninomiya, 1998). To investigate the formation of this differentially responsive neural system, our previous study examined recovery of NaCl responses and amiloride sensitivity of the regenerated nerve after the mouse CT nerve was disrupted by being crushed (nerve crush; Yasumatsu *et al.*, 2003). We found that responses to salts gradually reappeared 3 weeks after the nerve crush. At 3 weeks post crush almost all fibers responding to NaCl are amiloride-insensitive (AI: E-type). However, at 4 weeks some of the fibers showed amiloride sensitivity (AS: N-type), and after >5 weeks the number of N-type fibers reached the control level. During the course of recovery after CT crush, N-type and E-type fibers were clearly distinguishable on the basis of their amiloride sensitivities, their KCl/NaCl response ratios, and their concentration–response relationships to NaCl (Yasumatsu *et al.*, 2003). Thus these salt-responsive systems are apparently independently reformed during reinnervation. Also, the findings are consistent with the view that regenerating taste axons selectively innervate their corresponding classes of taste progenitor cells. In our previous study, however, we did not examine the molecular basis for the recovery of AS, and consequently it

remains unknown whether the salt receptor components are expressed in the progenitor cells prior to reinnervation.

ENaCs are known to consist of at least three subunits (α, β and γ), each of which posseses two transmembrane domains (Canessa *et al.*, 1993, 1994; Lingueglia *et al.*, 1993). The αENaC confers a low amplitude, amiloride-sensitive, sodium current, whereas β and γ subunits are required for the maximal channel activity. All three subunits of ENaC are reported to be expressed in rodent taste cells, with some tongue regional differences in their expression levels (Simon *et al.*, 1993; Li *et al.*, 1994, 1999; Kretz *et al.*, 1999). For example, cytoplasmic immunoreactivity for all three subunits was observed in nearly all taste cells of fungiform papillae whereas only about half of the taste cells of foliate and circumvallate papillae showed immunoreactivity. The intensity of labeling of circumvallate papilla cells was significantly lower than that of fungiform papilla cells, especially for the β and γ subunits (Lin *et al.*, 1999). A previous electrophysiological study using a patch-clamp analysis demonstrated that ~65% of the fungiform taste cells exhibited functional amiloride-sensitive Na^+ currents, whereas only 35% of the foliate taste cells were amiloride-sensitive. In contrast, taste cells of circumvallate papillae are completely amiloride-insensitive (Doolin and Gilbertson, 1996). Taken together, the presence of all three ENaC subunits in a taste receptor cells may be important in ensuring a functional AS cell.

It was the goal of this study, therefore, to explore the molecular mechanisms of recovery of AS after the CT nerve crush. To accomplish this, we examined the expression patterns of the three ENaC subunits (α, β and γ) in taste cells after crush of the CT nerve by using *in situ* hybridization (ISH) in mice.

Materials and methods

Experimental manipulation

All experimental procedures were approved by the committee for Laboratory Animal Care and Use at Kyushu University (Fukuoka, Japan). Subjects were adult male and female C57BL/6NCrj mice (Charles River Japan, Tokyo, Japan), 8–20 weeks of age, ranging in weight from 20 to 32 g. At 8–15 weeks of age, mice were divided into six groups, including one intact control group and five experimental (nerve-crush) groups with bilateral CT nerve crush. Five nerve-crush groups (1, 2, 3, 4 and ≥5 week groups) provided data at time points 7–9, 14–16, 21–23, 28–30 and ≥35 days (<50) after the CT nerve crush and regeneration. Each animal in each group was used for ISH analysis. For CT nerve crush, animals were anesthetized with pentobarbital sodium (40–50 mg/kg, i.p.). Bilateral CT nerves were exposed 5 mm rostrally apart from their entry to the bulla and repeatedly crushed at a single point with a number 5 forceps until only a thin strand of nerve sheath remained (5–10 crushes). We chose nerve crush instead of nerve section because in our pilot study we found that the time needed for recovery of taste responses after nerve section varied extensively among individuals whereas the time needed for recovery of taste responses after nerve crush was much more stable. Intact control animals received no manipulations.

cRNA probes for in situ hybridization

Each mouse was anesthetized with one i.p. injection of Nembutal (40–50 mg/kg), and then killed by cervical dislocation and its tongue quickly removed. After washing the tongue with a normal extracellular solution (NES; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES–NaOH, pH 7.4), 0.2 ml of NES containing 1.0 mg/ml elastase (Roche, Indianapolis, IN) was injected between the epithelium and muscle layers. After incubation in NES continuously bubbled with 95% O_2 and 5% CO_2 for 15–20 min at 26°C, the epithelial sheet containing fungiform papillae was peeled off from the underlying muscle. The epithelium was washed with NES, pinned serosal side up in a silicone dish, and incubated in divalent cation-free extracellular solution containing 2 mM EDTA for 20 min at room temperature. Taste buds were individually removed from fungiform papillae using a 100 μm transfer pipette. One hundred taste buds from the taste papillae were transferred to an Eppendorf tube containing 100 μl (1 vol) of lysis buffer (4 M guanidine thiocyanate, 25 mM trisodium citrate, pH 7.0, 0.5% sodium *N*-lauroyl sarcosine, 0.1 M 2-mercaptoethanol) and homogenized. Yeast transfer RNA (1.0 μg) was used as a carrier. Sequentially, 0.1 vol of 2 M Na-acetate (pH 4.0), 1 vol of acidic phenol and 0.2 vol of chloroform–isoamyl alcohol (49:1) were added, the mixture being vortexed after each addition. After incubation at 4°C for 15 min and centrifugation at high speed, the aqueous phase was recovered and RNA was precipitated with 1 vol of isopropanol. After centrifugation, the pellet was resuspended in 1 vol of lysis buffer and RNA was reprecipitated with isopropanol. After two washings with 75% ethanol, the pellet was dried and dissolved in water. A cDNA was generated by reverse transcription [oligo $(dT)_{12–18}$ primer] with the superscript preamplification system (GIBCO/BRL, Gaithersburg, MD). Polymerase chain reactions (PCRs) were carried out with an equivalent of 10 taste buds per reaction. Genomic DNA did not contribute to the signal as suggested by two protocols. In the first, RNA was treated in parallel in the presence and absence of reverse transcriptase, and the material was then used for PCR. In the absence of reverse transcriptase, there was no amplification of fragments having the expected size. Primers were chosen to span one or more introns in order to exclude confusion with amplified fragments from genomic DNA. PCRs led in this case to the amplification of two bands, either specific for the genomic DNA (characterized by a longer size owing to the presence of at least one intron) or for the reverse

transcribed complementary DNA. The primers used for DNA amplification were as follows: αENaC: 5′-CTAATGATG-CTGGACCACACC-3′ and 5′-AAAGCGTCTGTTCCG-TGATGC-3′ (GenBank accession number, the expected sizes of PCR products from mRNA: AF112185, 556 bp); βENaC: 5′-GCCAGTGAAGAAGTACCTCC-3′ and 5′-CCTGGGTGGCACTGGTGAA-3′ (AF112186, 632 bp); γENaC: 5′-AAGAATCTGCCAGTTCGAGGC-3′ and 5′-TACCACTCCTGGATGGCATTG-3′ (AF112187, 671 bp); α-gustducin: 5′-AGATGGGAAGTGGAATTAGTTCAG-A-3′ and 5′-GCTCAGAAGAGCCCACAGTCTTTGA-3′ (X65747, 1069 bp). PCR was performed on PE9700 with the following conditions: 95°C for 5 min (1 cycle); 94°C for 30 s, 58°C for 30 s, 72°C for 120 s (40 cycles); 72°C for 5 min (1 cycle). The PCR solution contains 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, each primer at 0.5 μM, 200 μM deoxyribonucleoside 5′-triphosphate (dNTP) and 0.05 unit/μl of Ex Taq polymerase (Takara Bio, Otsu, Japan). These DNA fragments were purified and cloned into the pGEM T-Easy vector (Promega, Madison, WI), which were confirmed by direct sequencing and digested with appropriate restriction enzymes. Biotin-labeled antisense RNA probes were generated by *in vitro* transcription using digoxigenin-RNA labeling mix and SP6 or T7 RNA polymerase (Roche).

In situ hybridization

ISH experiments were performed as described previously (Shigemura *et al.*, 2003, 2004). Frozen blocks of the dissected anterior and posterior parts of the tongue embedded in the OCT compound (Sakura Finetechnical, Tokyo, Japan) were sectioned into 5- to 7-μm-thick slices, which were mounted on silane-coated glass slides. The cryosections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, treated twice with 0.1% diethyl pyrocarbonate in PBS for 15 min, washed with 5 × sodium citrate (SSC) for 15 min at room temperature, and then pre-hybridized in hybridization buffer consisting of 50% formamide, 5 × SSC, 5 × Denhardt's solution, 500 μg/ml denatured salmon testis DNA, 250 μg/ml denatured baker's yeast tRNA and 1 mM dithiothreitol for 1 h at room temperature. Hybridization was carried out in a hybridization buffer to which was added 200 ng/ml antisense riboprobe for 18 h at 58°C. After hybridization, sections were washed twice in 5 × SSC for 5 min each and twice in 0.2 × SSC for 30 min each at 65°C. Subsequently, the sections were immersed in TBS, consisting of 50 mM Tris–HCl (pH 7.5) and 150 mM NaCl, for 5 min at room temperature, put in the blocking solution containing 0.5% blocking reagent (Roche) in TBS for 30 min, and incubated with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (AP) (1:400 dilution) in the blocking solution for 60 min at room temperature. After three washes of 5 min each in TNT buffer, consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.05% Tween20, sections were immersed in AP buffer consisting of 100 mM Tris–HCl (pH9.5), 100 mM NaCl, and 50 mM $MgCl_2$ for 5 min. The signals were developed using nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as chromogenic substrates. The reaction was then stopped by rinsing the slides in tris-EDTA (TE) buffer after which they were mounted. The signal specificities of mRNA for each gene in the taste tissues were tested by using a sense probe as a negative control.

Data analysis

In order to examine the size of taste bud and the number of cells expressing ENaCs and α-gustducin at each regenerating period, we measured the taste bud width and counted positive cells in each taste bud of the fungiform papilla in horizontal sections. Taste bud width was defined as the maximum diameter in serial horizontal sections of a taste bud and was measured by using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA). To exclude artifactual signals, cells showing a density signal greater than the mean + 2 SD of the density in taste cells in the negative control (using sense probes) were considered positive using Image-Pro Plus (Ver. 4.0, Media Cybernetics Inc., Silver Spring, MD). All data are presented as mean ± SE. Significant differences in the number positive cells between six groups (control and nerve crush groups of 1, 2, 3, 4 and 5 weeks) were determined by one-way analysis of variance (ANOVA), followed by comparison of individual pairs of means using the *t*-test as post-hoc (StatView 5.0 program, Abacus Concepts, Berkeley, CA). A *P*-value of <0.05 was considered statistically significant.

Results

Antisense riboprobes specific for α, β and γENaC subunit mRNAs were applied to horizontal sections of the mouse fungiform papilla after the CT nerve crush. As shown in Figure 1, the α, β and γENaC subunit mRNAs were detected in some cells in taste buds of the fungiform papillae throughout the period tested (1–5 weeks). In the circumvallate and fungiform papillae of intact animals, αENaC mRNA was clearly and strongly detected. In contrast, the intensity of labeling for the β and γ subunits in taste cells was much lower in circumvallate than in fungiform papillae. This differential expression pattern of the three ENaC subunits in fungiform compared with circumvallate papillae was consistent with the pattern previously reported in rats (Kretz *et al.*, 1999; Lin *et al.*, 1999). Hybridization signals for all three ENaC subunits were also observed in very low abundance in non-taste epithelial cells in the anterior tongue (Figure 1).

ANOVA for taste bud width demonstrated significant difference among control, 1, 2, 3, 4 and 5 weeks after the CT nerve crush [$F(5,394) = 97.34$, $P < 0.0001$]. *Post hoc t*-tests indicated significant differences at 1 and 2 weeks (versus control, 3 weeks, 4 weeks or 5 weeks, $P < 0.01$) after the CT nerve crush. In contrast, no significant difference was observed at 1 week (versus 2 weeks, $P > 0.05$), 3 weeks (versus control,

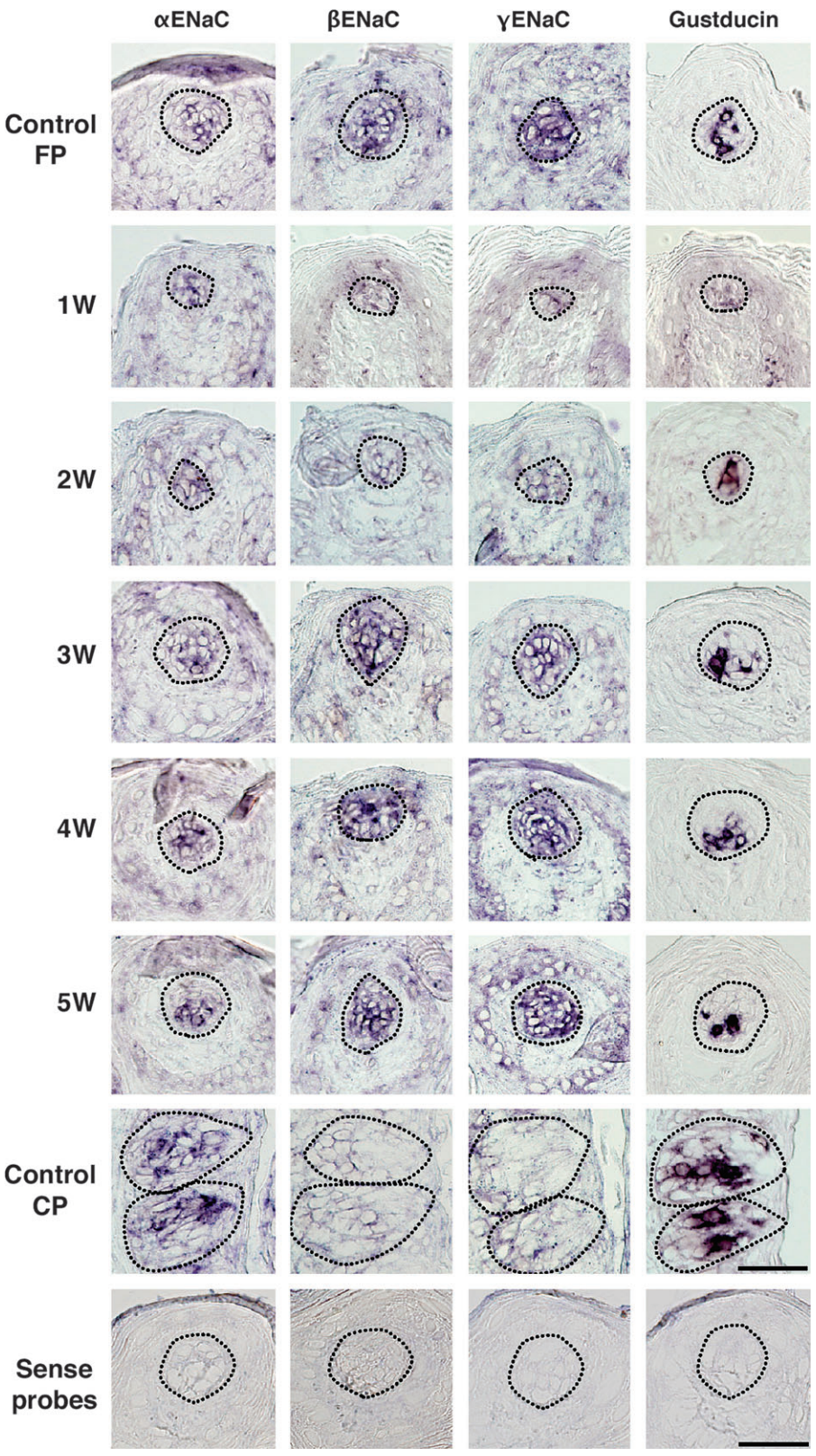

Figure 1 *In situ* hybridization analysis of α, β and γ amiloride-sensitive epithelial Na^+ channel (ENaC) subunits and α-gustducin mRNAs in fungiform (FP) and circumvallate papillae (CP) of intact control mice and FP of experimental mice at 1, 2, 3, 4 and 5 weeks after bilateral chorda tympani nerve crush. Taste bud cells at an early stage (1–2 weeks after nerve crush) already express α, β and γ subunits of ENaC or α-gustducin. The dotted lines indicate the outlines of sample taste buds. Bar, 50 μm.

4 weeks or 5 weeks, $P > 0.05$), 4 weeks (versus control or 5 weeks, $P > 0.05$) and 5 weeks (versus control, $P > 0.05$). Thus, the mean taste bud width reached the control level at 3 weeks after the CT nerve crush (Figure 2).

At 1 week after CT nerve crush, taste buds in fungiform papillae were reduced in the size and number. Some shrunken fungiform papillae without taste buds were also detected (data not shown). Although the size of taste bud was reduced (35.22 ± 0.75 μm, $P < 0.01$ versus 52.01 ± 1.55 in control), a few positive taste cells for all three ENaC subunits were detected at this period. α-Gustducin mRNA, a standard marker of taste cells (McLaughlin *et al.*, 1992), was also expressed in the shrunken taste buds. The intensity of the signals for all ENaC subunits and α-gustducin were weaker than those of intact control mice. At 2 weeks, the regenerating taste bud increased somewhat in the size in fungiform papilla (36.27 ± 0.74 μm, $P < 0.01$ versus control) and the intensity of signals for all ENaC subunits increased. At 3 weeks, the size of taste buds increased (49.58 ± 0.59 μm, $P > 0.05$ versus control) and the hybridization signal for all ENaC subunits was more clearly detected. At 4 and 5 weeks, the expression patterns for all ENaCs and for α-gustducin were almost same as those at 3 weeks after the CT nerve crush. The staining in controls using a sense probe was negative.

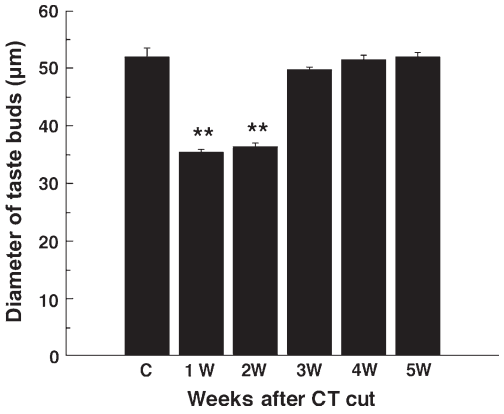

Figure 2 The mean diameters of taste buds in fungiform papillae (FP) of the intact control ($n = 25$ buds) and experimental groups at 1 ($n = 73$), 2 ($n = 98$), 3 ($n = 97$), 4 ($n = 75$) and 5 ($n = 32$) weeks after bilateral chorda tympani (CT) nerve crush. Significant differences in the mean diameters among the six groups (control, 1–5 weeks) in FP were determined by one way ANOVA, followed by comparison of individual pairs of means using *t*-tests. $^{**}P < 0.01$. The size of taste buds in FP reached the control level at 3 weeks post-surgery. C, control group; W, weeks after the CT nerve crush.

To determine the number of cells expressing ENaCs and α-gustducin at each regeneration periods, we counted positive cells for every taste bud in every 50 μm horizontal section of the anterior tongue of each animal. The mean numbers of ENaCs and α-gustducin positive cells in fungiform and circumvallate papillae are shown in Figure 3 and Table 1. Significant differences between regeneration periods were determined by one factor ANOVA, followed by a comparison of individual pairs of means using *t*-tests. Each ANOVA for α, β and γENaC and gustducin demonstrated significant difference among control, 1, 2, 3, 4 and 5 weeks after the CT nerve crush [$F(5,168–182) = 7.20–17.94$, $P < 0.0001$]. *Post hoc* *t*-tests for each gene indicated significant differences in the mean number of positive cells at 1 week (versus control, 3 weeks, 4 weeks or 5 weeks, $P < 0.01$) and 2 weeks (versus control, 3 weeks, 4 weeks or 5 weeks, $P < 0.05$) after the CT nerve crush. In contrast, no significant difference was observed at 1 week (versus 2 weeks, $P > 0.05$), 3 weeks (versus control, 4 weeks or 5 weeks, $P > 0.05$), 4 weeks (versus control or 5 weeks, $P > 0.05$) and 5 weeks (versus control, $P > 0.05$). Thus, the mean number of cells per a taste bud that express each ENaC subunits or α-gustducin reached the control level at 3 weeks after the CT nerve crush.

Discussion

In the present study, we investigated molecular mechanisms underlying recovery of salt receptor systems after nerve crush by examining expression patterns of each of the three subunits of ENaC (α, β and γ) in the fungiform papillae by using

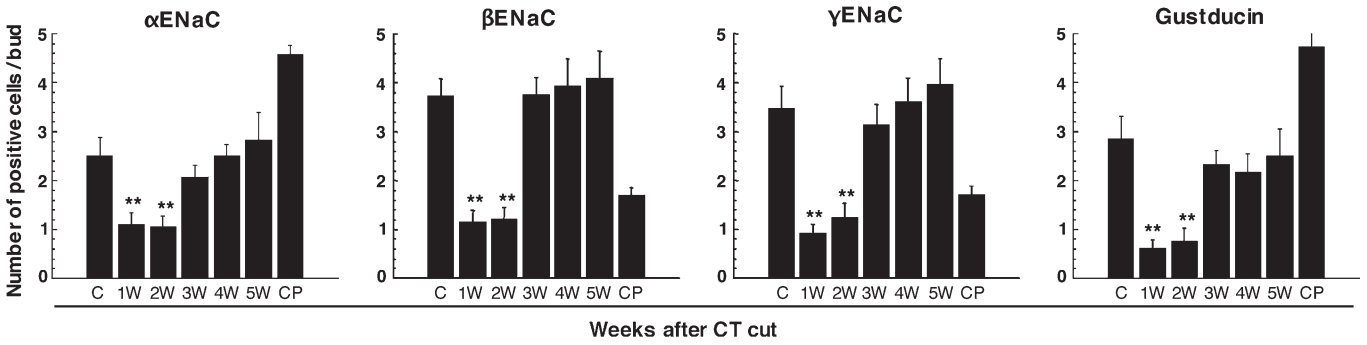

Figure 3 The mean number of positive cells per a taste bud in fungiform (FP) and circumvallate papillae (CP) of the intact control group ($n = 18–26$ buds for each gene) and FP of experimental groups at 1 ($n = 40–44$), 2 ($n = 26–38$), 3 ($n = 34–45$), 4 ($n = 23–32$) and 5 ($n = 11–18$) weeks after bilateral chorda tympani (CT) nerve crush. Significant differences in the mean number of the positive cells among the six groups (control, 1–5 weeks) in FP were determined by one way ANOVA, followed by comparison of individual pairs of means using *t*-tests. $^{**}P < 0.01$. The number of cells per a taste bud expressing each ENaC subunit or α-gustducin reached the control level at 3 weeks. C, control group; W, weeks after the CT nerve crush.

Table 1 Mean number ± SE of cells expressing ENaCs and gustducin in a fungiform papilla after CT cut (*n* = the number of taste buds examined)

Genes	FP before CT cut	Weeks after CT cut					CP before CT cut
		1	2	3	4	5	
αENaC	2.50 ± 0.38 (*n* = 18)	1.10 ± 0.24** (*n* = 41)	1.05 ± 0.22** (*n* = 38)	2.06 ± 0.25 (*n* = 34)	2.50 ± 0.24 (*n* = 32)	2.82 ± 0.57 (*n* = 11)	4.57 ± 0.20 (*n* = 35)
βENaC	3.71 ± 0.36 (*n* = 21)	1.14 ± 0.25** (*n* = 42)	1.20 ± 0.24** (*n* = 30)	3.76 ± 0.35 (*n* = 45)	3.92 ± 0.34 (*n* = 25)	4.08 ± 0.57 (*n* = 12)	1.67 ± 0.17 (*n* = 47)
γENaC	3.46 ± 0.46 (*n* = 26)	0.90 ± 0.20** (*n* = 40)	1.23 ± 0.31** (*n* = 34)	3.14 ± 0.41 (*n* = 37)	3.59 ± 0.50 (*n* = 32)	3.94 ± 0.54 (*n* = 18)	1.72 ± 0.17 (*n* = 54)
Gustducin	2.86 ± 0.46 (*n* = 21)	0.61 ± 0.18** (*n* = 44)	0.77 ± 0.25** (*n* = 26)	2.33 ± 0.29 (*n* = 36)	2.17 ± 0.38 (*n* = 23)	2.50 ± 0.56 (*n* = 16)	4.74 ± 0.31 (*n* = 44)

**$P < 0.01$ versus before CT cut. Significant differences between regeneration terms were determined by ANOVA, followed by a comparison of individual pairs of means using the *t*-test.

ISH analysis. The results suggest that all three ENaC subunits were expressed in a subset of taste bud cells from an early stage (1–2 weeks) after CT nerve crush, although taste buds were smaller and fewer than those of control mice at this stage. Previous histological studies of taste buds after taste nerve crush showed that ~80–85% of the total number of taste buds degenerated within the first 2 weeks after interruption of the CT nerve, with the residual taste buds classified into two groups: atrophic taste buds and taste bud remnants (Oakley *et al.*, 1993). A few taste cells in the atrophic taste buds express keratin 19 in gerbils, hamsters and rats (Oakley *et al.*, 1993; McCluskey and Hill, 2002), and neural cell adhesion model (NCAM) neuron-specific enolase and calcitonin gene-related peptide (CGRP) in hamsters (Whitehead *et al.*, 1998), which are considered as markers of differentiated taste cells. A recent study in mice also reported that the signal intensity of Mash1, Nkx2.2, T1R3 and gustducin gradually decreased in taste buds until 6 days after bilateral IXth nerve crush, whereas the expression of sonic hedgehog (shh) in the basal cells and patched1 (ptc) around taste buds quickly disappeared within 12 h after the nerve crush. The shh expression reappeared immediately after the regenerated nerve reached the taste bud, and NCAM expression in regenerated vallate taste cells near the shh-expressing cells was detected at 11 days after the nerve crush (Miura *et al.*, 2004). Comparably, in rats, NCAM immnoreactivity reappeared at 10–12 days after bilateral IXth nerve crush (Smith *et al.*, 1994).

Our previous electrophysiological study showed that robust responses to anodal currents and cold stimulations were observed at 2 weeks after nerve crush. This implies that the regenerated CT at this stage might have reached the epithelial tissue just below the tongue surface, although no chemically responsive elements of taste cells were restored as yet (Yasumatsu *et al.*, 2003). Taken together, the expression of ENaC subunits observed at an early stage (1–2 weeks) after CT nerve crush in the present study may be due to the existence of at least two types of taste cells. One type may be surviving taste cells which have differentiated and expressed ENaC subunits before the nerve crush. Another type may be taste cells that regenerated after the crush. At this time, both surviving and regenerated taste cells expressing ENaCs may not reconnect with AS or AI fibers of the CT nerve because no chemical response of the CT nerve was observed (Yasumatsu *et al.*, 2003). The ENaC expression in the regenerated cells may reflect the reappearance of shh-expressing cells that are in contact with the regenerated nerve in the base of the taste buds, although the details remain unclear.

Immunoreactivity of amiloride-sensitive Na^+ channels has been observed in rat taste cells of fungiform papilla taste buds as early as the first day after birth (Stewart *et al.*, 1995), although the rat CT nerve does not exhibit significant amiloride sensitivity before 12–13 postnatal days (Hill and Bour, 1985). Kossel *et al.* (1997) showed that single taste cells of fungiform papilla taste buds from rats as young as 2 postnatal days exhibit amiloride-blockable currents. Furthermore, Mbiene and Roberts (2003) showed that there are more embryonic taste buds generated before innervation (37.7%) than after nerve contacts with the lingual epithelium were established (32.2%). These data for taste bud development are consistent with our finding of ENaC expressing taste receptor cells without synapses at an early stage following nerve crush.

We also showed that the mean number of cells per taste bud that expressed each ENaC subunit or α-gustducin and the size of taste buds reached control levels at 3 weeks after CT nerve crush. Our previous electrophysiological study demonstrated that by 3 weeks after nerve crush responses to salts had reappeared, although NaCl responses were not yet inhibited by amiloride (only AI response) (Yasumatsu *et al.*, 2003). As shown by the shrinkage of taste buds after the CT nerve crush, factors specific to gustatory nerves likely provide important trophic interactions with taste cells. Therefore, the rapid increase in both the size of taste buds and in the number of ENaC subunit-positive cells may be caused by reinnervation at this stage.

The differential expression patterns of ENaCs in fungiform and circumvallate papillae of control intact mice were comparable with previous immunohistochemical and PCR

studies using rats (Kretz *et al.*, 1999; Lin *et al.*, 1999). That is, all three ENaC subunits were abundantly present in taste cells in fungiform papillae, whereas the intensity of labeling for β and γ subunits was clearly lower than that for αENaC in circumvallate papillae. The IXth nerve, innervating a circumvallate papilla, has primarily the E type (amiloride-insensitive or AI fibers), showing only the AI component of the NaCl response (Formaker and Hill, 1991; Ninomiya *et al.*, 1991; Ninomiya, 1998). If unequal expression levels of the three ENaC subunits, as shown in circumvallate papillae, are involved in occurrence of the AI response component, it is possible that a similarly unequal expression of the three subunits may appear at 3 weeks after the CT crush. However, no difference in expression levels of three subunits was observed in fungiform papillae. It is proposed that AI cells and fibers are sensitive to various electolytes (salts and acids) and that they may have multiple receptor mechanisms [i.e. apical K^+ channel (Kinnamon *et al.*, 1988), non-specific cation channels (Miyamoto *et al.*, 1998), inwardly rectifying Cl^- channel protein, CIC-2 (Miyamoto *et al.*, 2001), hyperpolarization-activated cyclic nucleotide-gated cation channels (Stevens *et al.*, 2001) and vanilloid receptor-1 variant (Lyall *et al.*, 2004)]. Another transduction system for AI response begins with electoroneutral diffusion of the salt across the tight junctions between taste receptor cells and sodium entry into the cells via unspecified basolateral ion channels (Elliott and Simon, 1990; Ye *et al.*, 1991, 1993). This paracellular pathway would not be blocked by mucosal application of amiloride and would allow entry of small ions, such as Na^+, K^+, H^+ and Cl^-, which may thus provoke AI cell responses. Therefore, at this stage, one suggestion to account for the mechanism of AI response may be that all three ENaC subunits are situated only basolaterally. At 4 weeks after the CT crush, the reappearance of an AS response (Yasumatsu, *et al.*, 2003) may be caused by the reinnervation of AS axons with the taste cell permitting redistribution of fully functional channels from the basolateral to the apical domain. In rats, the redistribution of the channels in taste cells is proposed to be involved in the developmental increase in neural amiloride sensitivity (Kossel *et al.*, 1997). If this is the case, however, different patterns of the three subunits between fungiform and circumvallate papillae, suggested by previous reports (Kretz *et al.*, 1999; Lin *et al.*, 1999), may not be involved in tongue regional difference in amiloride sensitivity.

In conclusion, we found that taste bud cells at an early stage (1–2 weeks) after nerve crush express α, β and γ subunits of ENaC. The number of cells per a taste bud that expressed each ENaC subunit or α-gustducin and the size of taste buds reached control levels at 3 weeks after nerve crush, when salt-responsive neural responses also reappeared. This suggests that differentiation of taste cells into AS or AI cells may start before synapse formation. The earlier expression and functional maturation of ENaC subunits in taste cells may, therefore, increase the capability of regenerated axons to selectively innervate their matched AS and AI salt responsive cells, as suggested by our previous study (Yasumatsu *et al.*, 2003). In order to clarify the mechanism for formation of functional salt-responsive systems, however, future examination of both physiological taste responses and gene expression profiles for ENaC and other related molecules in each AS and AI taste cells is needed.

Acknowledgements

We thank Dr Gary K. Beauchamp for his valuable comments and suggestions on the manuscript. This research was supported in part by a Grant-in-Aids 15209061 (Y.N.) for Scientific Research from Japan Society for the promotion of science.

References

Canessa, C.M., Horisberger, J.D. and **Rossier, B.C.** (1993) *Epithelial sodium channel related to proteins involved in neurodegeneration.* Nature, 361, 467–470.

Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J.D. and **Rossier, B.C.** (1994) *Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits.* Nature, 367, 463–467.

Doolin, R.E. and **Gilbertson, T.A.** (1996) *Distribution and characterization of functional amiloride-sensitive sodium channels in rat tongue.* J. Gen. Physiol., 107, 545–554.

Elliott, E.J. and **Simon, S.A.** (1990) *The anion in salt taste: a possible role for paracellular pathways.* Brain Res., 535, 9–17.

Formaker, B.K. and **Hill, D.L.** (1991) *Lack of amiloride sensitivity in SHR and WKY glossopharyngeal taste responses to NaCl.* Physiol. Behav., 50, 765–769.

Hettinger, T.P. and **Frank, M.E.** (1990) *Specificity of amiloride inhibition of hamster taste responses.* Brain Res., 513, 24–34.

Hill, D.L. and **Bour, T.C.** (1985) *Addition of functional amiloride-sensitive components to the receptor membrane: a possible mechanism for altered taste responses during development.* Brain Res., 352, 310–313.

Kinnamon, S.C., Dionne, V.E. and **Beam, K.G.** (1988) *Apical localization of K^+ channels in taste cells provides the basis for sour taste transduction.* Proc. Natl Acad. Sci. USA, 85, 7023–7027.

Kossel, A.H., McPheeters, M., Lin, W. and **Kinnamon, S.C.** (1997) *Development of membrane properties in taste cells of fungiform papillae: functional evidence for early presence of amiloride-sensitive sodium channels.* J. Neurosci., 17, 9634–9641.

Kretz, O., Barbry, P., Bock, R. and **Lindemann, B.** (1999) *Differential expression of RNA and protein of the three pore-forming subunits of the amiloride-sensitive epithelial sodium channel in taste buds of the rat.* J. Histochem. Cytochem., 47, 51–64.

Li, X.J., Blackshaw, S. and **Snyder, S.H.** (1994) *Expression and localization of amiloride-sensitive sodium channel indicate a role for non-taste cells in taste perception.* Proc. Natl Acad. Sci. USA, 91, 1814–1818.

Lin, W., Finger, T.E., Rossier, B.C. and **Kinnamon, S.C.** (1999) *Epithelial Na^+ channel subunits in rat taste cells: localization and regulation by aldosterone.* J. Comp. Neurol., 405, 406–420.

Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M. and **Barbry, P.** (1993) *Expression cloning of an epithelial amiloride-sensitive Na^+ channel. A new channel type with homologies to Caenorhabditis elegans degenerins.* FEBS Lett., 318, 95–99.

Lyall, V., Heck, G.L., Vinnikova, A.K., Ghosh, S., Phan, T.H., Alam, R.I., Russell, O.F., Malik, S.A., Bigbee, J.W. and **DeSimone, J.A.** (2004) *The mammalian amiloride-insensitive non-specific salt taste receptor is a vanilloid receptor-1 variant.* J. Physiol., 558, 147–159.

Mbiene, J.P. and **Roberts, J.D.** (2003) *Distribution of keratin 8-containing cell clusters in mouse embryonic tongue: evidence for a prepattern for taste bud development.* J. Comp. Neurol., 457, 111–122.

McCluskey, L.P. and **Hill, D.L.** (2002) *Sensitive periods for the effect of dietary sodium restriction on intact and denervated taste receptor cells.* Am. J. Physiol. Regul. Integr. Comp. Physiol., 283, R1275–1284.

McLaughlin, S.K., McKinnon, P.J. and **Margolskee, R.F.** (1992) *Gustducin is a taste-cell-specific G protein closely related to the transducins.* Nature, 357, 563–569.

Miura, H., Kato, H., Kusakabe, Y., Tagami, M., Miura-Ohnuma, J., Ninomiya, Y. and **Hino, A.** (2004) *A strong nerve dependence of Sonic hedghog expression in basal cells in mouse taste bud and an autonomous transcriptional control of genes in differentiated taste cells.* Chem. Senses, 29, 823–831.

Miyamoto, T., Fujiyama, R., Okada, Y. and **Sato, T.** (1998) *Sour transduction involves activation of NPPB-sensitive conductance in mouse taste cells.* J. Neurophysiol., 80, 1852–1859.

Miyamoto, T., Miyazaki, T., Fujiyama, R., Okada, Y. and **Sato, T.** (2001) *Differential transduction mechanisms underlying NaCl- and KCl-induced responses in mouse taste cells.* Chem. Senses, 26, 67–77.

Ninomiya, Y. (1996) *Salt taste responses of mouse chorda tympani neurons: evidence for existence of two different amiloride-sensitive receptor components for NaCl with different temperature dependencies.* J. Neurophysiol., 76, 3550–3554.

Ninomiya, Y. (1998) *Reinnervation of cross-regenerated gustatory nerve fibers into amiloride-sensitive and amiloride-insensitive taste receptor cells.* Proc. Natl Acad. Sci. USA, 95, 5347–5350.

Ninomiya, Y. and **Funakoshi, M.** (1988) *Amiloride inhibition of responses of rat single chorda tympani fibers to chemical and electrical tongue stimulations.* Brain Res., 451, 319–25.

Ninomiya, Y., Tanimukai, T., Yoshida, S. and **Funakoshi, M.** (1991) *Gustatory neural responses in preweanling mice.* Physiol. Behav., 49, 913–918.

Oakley, B., Lawton, A., Riddle, D.R. and **Wu, L.H.** (1993) *Morphometric and immunocytochemical assessment of fungiform taste buds after interruption of the chorda-lingual nerve.* Microsc. Res. Tech., 26, 187–195.

Shigemura, N., Miura, H., Kusakabe, Y., Hino, A. and **Ninomiya, Y.** (2003) *Expression of leptin receptor (Ob-R) isoforms and signal transducers and activators of transcription (STATs) mRNAs in the mouse taste buds.* Arch. Histol. Cytol., 66, 253–260.

Shigemura, N., Ohta, R., Kusakabe, Y., Miura, H., Hino, A., Koyano, K., Nakashima, K. and **Ninomiya, Y.** (2004) *Leptin modulates behavioral responses to sweet substances by influencing peripheral taste structures.* Endocrinology, 145, 839–847.

Simon, S.A., Holland, V.F., Benos, D.J. and **Zampighi, G.A.** (1993) *Transcellular and paracellular pathways in lingual epithelia and their influence in taste transduction.* Microsc. Res. Tech., 26, 196–208.

Smith, D.V., Klevitsky, R., Akeson, R.A. and **Shipley, M.T.** (1994) *Expression of the neural cell adhesion molecule (NCAM) and polysialic acid during taste bud degeneration and regeneration.* J. Comp. Neurol., 347, 187–196.

Stevens, D.R., Seifert, R., Bufe, B., Muller, F., Kremmer, E., Gauss, R., Meyerhof, W., Kaupp, U.B. and **Lindemann, B.** (2001) *Hyperpolarization-activated channels HCN1 and HCN4 mediate responses to sour stimuli.* Nature., 413, 631–635.

Stewart, R.E., Lasiter, P.S., Benos, D.J. and **Hill, D.L.** (1995) *Immunohistochemical correlates of peripheral gustatory sensitivity to sodium and amiloride.* Acta Anat., 153, 310–319.

Whitehead, M.C., Ganchrow, J.R., Ganchrow, D. and **Yao, B.** (1998) *Neural cell adhesion molecule, neuron-specific enolase and calcitonin gene-related peptide immunoreactivity in hamster taste buds after chorda tympani/lingual nerve denervation.* Neuroscience, 83, 843–856.

Yasumatsu, K., Katsukawa, H., Sasamoto, K. and **Ninomiya, Y.** (2003) *Recovery of amiloride-sensitive neural coding during regeneration of the gustatory nerve: behavioral–neural correlation of salt taste discrimination.* J. Neurosci., 23, 4362–4368.

Ye, Q., Heck, G.L. and **DeSimone, J.A.** (1991) *The anion paradox in sodium taste reception: resolution by voltage-clamp studies.* Science., 254, 724–726.

Ye, Q., Heck, G.L. and **DeSimone, J.A.** (1993) *Voltage dependence of the rat chorda tympani response to Na^+ salts: implications for the functional organization of taste receptor cells.* J. Neurophysiol., 70, 167–178.

Accepted June 26, 2005