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Electrophysiological Heterogeneity in a Functional Subset of Mouse Taste Cells during Postnatal Development

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Abstract

Taste cells in adult mammals are functionally heterogeneous as to the expression of ion channels. How these adult phenotypes are established during postnatal development, however, is not yet clear. We have addressed this issue by studying voltage-gated K^+ and Cl^- currents (I_K and I_{Cl}, respectively) in developing taste cells of the mouse vallate papilla. I_K and I_{Cl} underlie action potential waveform and firing properties, and play an important role in taste transduction. By using the patch clamp technique, we analyzed these currents in a specific group of cells, called Na/OUT cells and thought to be sensory. In adult mice, three different electrophysiological phenotypes of Na/OUT cells could be detected: cells with I_K (K cells); cells with both I_K and I_{Cl} (K+Cl cells); and cells with I_{Cl} (Cl cells). In contrast, at early developmental stages (2–4 postnatal days, PD) there were no Cl cells, which appeared at PD 8. Our findings indicate a mechanism that contributes to building-up the functional heterogeneity of mammalian taste cells during the postnatal development.

Key words: ion currents, gustatory, patch-clamp, vallate papilla

Introduction

In adult mammals, taste cells possess quite complex membrane properties due to the presence of ion channels. Among them, voltage-gated ones play an important role in sensory transduction. For example, they underlie the generation of action potentials (e.g. Herness and Sun, 1995; Chen *et al.*, 1996), which are thought to mediate signal processing inside taste buds during chemical stimulation (e.g. Varkevisser *et al.*, 2001). In addition, they are targeted by gustatory stimuli (Cummings *et al.*, 1996; Chen and Herness, 1997; Gilbertson *et al.*, 1997; Zhao *et al.*, 2002; Park *et al.*, 2003) as well as by hormones and neuromodulators (Herness and Chen, 2000; Kawai *et al.*, 2000; Herness *et al.*, 2002a,b). Thus, voltage-gated channels are key molecules in taste reception.

One interesting aspect of the biology of taste cells is that they are functionally heterogenous in regard to the expression of voltage-gated ion channels (e.g. Akabas *et al.*, 1990; Béhé *et al.*, 1990; Chen *et al.*, 1996; Kossel *et al.*, 1997; Bigiani, 2001; Bigiani *et al.*, 2002; Medler *et al.*, 2003). It is not known yet how the 'adult' phenotypes are established during taste cell maturation, which, at least in part, takes place after birth in precocial mammals, such as rodents (reviewed in Mistretta and Hill, 1995; Stewart *et al.*, 1997; Barlow, 2000). Recently, we have provided evidence that electrophysiological heterogeneity of mouse taste cells changes with postnatal age (Bigiani *et al.*, 2002). For example, spiking cells appear at early stages of development, whereas non-spiking, glia-like elements can be detected after the second postnatal week. Given the role of ion channels in taste transduction, information on the development of the functional phenotypes may provide insights into the changes of chemosensory properties that occur before adulthood (reviewed in Mistretta and Hill, 1995; Stewart *et al.*, 1997).

In this study, we have addressed the issue of functional maturation of taste cells during postnatal development. Specifically, we wanted to gain further information on the electrophysiological heterogeneity for voltage-gated ion channels in developing taste cells. By applying the patch-clamp technique to single cells in taste buds isolated from the mouse vallate papilla, we have studied the occurrence of voltage-gated K^+ and Cl^- currents (I_K and I_{Cl}, respectively) in a well-defined subset of taste cells [called Na/OUT cells, thought to be sensory cells (Bigiani *et al.*, 2002)] throughout the postnatal development. According to the relative contributions of I_K and I_{Cl} to the outward currents in Na/OUT cells, we have been able to evaluate their electrophysiological heterogeneity at given postnatal ages and how it changed after birth. Our findings indicate that three main

subtypes of Na/OUT cells can be identified in adult mice and that one of them appears belatedly with respect to the others during postnatal development.

Materials and methods

Tissue preparation

CD-1 mice were used. Vallate taste buds were isolated with an enzymatic–mechanical procedure we described previously (Bigiani *et al.*, 2002). Briefly, mice were deeply anesthetized by CO_2 and killed by dislocation of cervical vertebrae. Tongues were rapidly removed and placed in Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, 10 Na pyruvate, pH 7.4 with NaOH). Two milligrams of elastase (Worthington Biochemical Corporation, Freehold, NJ) and 2 mg of dispase (grade II; Boehringer Mannheim, Germany) in 1.0 ml of Tyrode solution were injected (0.2–0.4 ml/tongue) between the lingual epithelium and muscle layer. Tongues were incubated in Ca^{2+}-free Tyrode solution at 30°C for ~15–70 min, depending on the age of the animal [incubations of longer duration were required for juveniles (Kossel *et al.*, 1997; Bigiani *et al.*, 2002)]. As a control, we checked that long incubation times did not affect the membrane properties of taste cells in adult mice. After incubation, the lingual epithelium could be peeled free from the underlying tissue with gentle dissection. The freed epithelium was pinned serosal side up in a Sylgard-lined petri dish and incubated in Ca^{2+}-free Tyrode solution for ~5–10 min to loosen the attachments of taste buds to the papilla. Vallate taste buds were removed from the epithelium by gentle suction with a fire-polished pipette (tip diameter ~50–100 μm) and plated on the bottom of a chamber that consisted of a standard glass slide onto which a silicon ring (1–2 mm thick and 15 mm internal diameter) was pressed. The glass slide was precoated with Cell-Tak (~3 μg/cm^2; Collaborative Research, Bedford, MA) to improve adherence of isolated taste buds to the bottom of the chamber. The chamber was placed on the stage of an inverted Olympus microscope (IX70; Olympus, Tokyo, Japan) and taste buds were viewed with Nomarski optics at 750×. During the experiments, isolated taste buds were continuously perfused with Tyrode solution (flow rate: 2–3 ml/min) by means of a gravity-driven system. Drugs were dissolved in modified Tyrode solutions to maintain osmolarity. All chemicals were from Sigma Chemical Co. (St Louis, MO).

Recording techniques

Membrane currents of single cells in isolated taste buds were studied at room temperature (22–25°C) by whole-cell patch-clamp (Hamill *et al.*, 1981), using an Axopatch 1D amplifier (Axon Instruments, Union City, CA). Signals were recorded and analyzed using a Pentium computer equipped with Digidata 1320 data acquisition system and pClamp8 software (Axon Instruments). pClamp8 was used to generate voltage-clamp commands and to record the resulting data. Signals were prefiltered at 5 kHz and digitized at 50 μs intervals.

Patch pipettes were made from soda lime glass capillaries (Baxter Scientific Products, McGaw Park, IL) on a two-stage vertical puller (PP-830; Narishige, Tokyo, Japan). The standard pipette solution contained (in mM): 120 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 11 EGTA, 2 ATP, 0.4 GTP, pH 7.3 with KOH. In some experiments, KCl was replaced by an equal concentration of K gluconate. Pipette resistances typically were 3–5 MΩ when filled with intracellular solution. The access resistance of the patch pipette tip was estimated by dividing the amplitude of the voltage steps by the peak of the capacitive transients (from which stray capacitance had been subtracted). Values typically ranged from ~8 to 15 MΩ. Leakage and capacitive currents were not subtracted from currents under voltage clamp and all voltages have been corrected for liquid junction potential (LJP; ~4 mV for KCl pipette solution and ~10 mV for K gluconate pipette solution) measured between pipette solution and Tyrode (bath) solution (Neher, 1992).

Analysis of electrophysiological data

The quality of the patch electrode seal and the state of the recorded cell can affect electrophysiological recordings (Barry and Lynch, 1991). Accordingly, in this study we analyzed only data from those taste cells that fulfilled the following criteria: (i) seal resistance > 5 GΩ and (ii) input resistance (R_{in}) in whole-cell configuration > 1 GΩ. In fact, a smaller R_{in} could be either a sign of 'leaky' seal or of recording from a glia-like taste cell (Bigiani, 2001).

Most data analysis was performed using pClamp8. Additional analysis and plotting were performed using Prism 3.03 software (GraphPad Software, San Diego, CA). Results are presented as means ± standard error of the means (SEM). Data comparisons were made with a two-tailed independent *t*-test for Gaussian distributions. We also used the Mann–Whitney (non-parametric) test for comparing the medians of skewed distributions. Significance level was taken as $P < 0.05$. Distribution of experimental data was displayed in the form of a box plot. In this plot, boxes show the middle half of the data (between the 25th and 75th percentiles) and the horizontal line marks the median, whereas the 'whiskers' extending from the top and bottom of the boxes show the main body of the data (that is, those that are within 3/2 times the interquartile range of data included in the box). Outliers or extreme values are plotted individually with circles. Note that the median is less sensitive to extreme scores than the mean and this makes it a better measure than the mean for skewed distributions (Chambers *et al.*, 1983).

Results

Contribution of I_K and I_{Cl} to the outward currents in Na/OUT cells during postnatal development

In this study, we focused our attention to one specific group of cells characterized by the expression of voltage-gated K^+ and Cl^- currents (I_K and I_{Cl}, respectively), in addition to voltage-gated Na^+ current. These cells have been called 'Na/OUT' cells, and are thought to be sensory in function (Bigiani, 2001; Bigiani *et al.*, 2002). Both I_K and I_{Cl} were 'outward' currents under our ionic conditions (Fig. 1*A*). However, the relative contribution of I_K and I_{Cl} to the whole outward current varied conspicuously from cell to cell. The pattern of this variability was visualized by testing the effect of tetraethylammonium (TEA, a potassium channel blocker; Rudy, 1988) on the outward current, and then by evaluating the distribution of the TEA block among recorded cells at given ages. This was possible because the current remaining during TEA block was a chloride current, as demonstrated by its sensitivity to 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, a known Cl^- conductance blocker in taste cells; Taylor and Roper, 1994; Herness and Sun, 1999; Bigiani *et al.*, 2002) or by its complete suppression in chloride-free solution (Fig. 1*A*). In short, a strong TEA effect indicated that the tested cell was endowed with a large I_K component, whereas a weak TEA effect indicated that the tested cell possessed a large I_{Cl} component.

Figure 1*B* shows the distribution of the effect of TEA on the outward currents recorded from taste cells of mice at specific postnatal ages (PD 2, 3, 4, 8, 14, 21) and of adult mice (age range: 40–80 days). The presence of sodium currents was used as a functional marker to identify Na/OUT cells throughout the postnatal development. The distribution of cells at each age displayed in the form of a box plot (Fig. 1*B*) provides an excellent tool for detecting and illustrating age-related changes. The box describes the number of cells that falls in the middle of the distribution (between the 25th and 75th percentiles). The median is the middle of the distribution and is shown as a horizontal line across the box. The main body of the data is indicated by the lower and the upper whiskers. Finally, outliers are represented by circles. It was clear from the box plots that the pattern of diversification for outward currents changed considerably during development (Fig. 1*B*). The large leap in the functional diversification occurred during the second week (between PD 8 and PD 14): this could be appreciated by observing the position of the box, which was always situated in the uppermost part of the plot during the first week, whereas it gradually broadened toward the bottom of the plot after PD 8. Comparison with the Mann–Whitney test showed that the distribution at PD 2–8 differed significantly from that in adult ($P < 0.001$). On the contrary, distribution at PD 14 and PD 21 did not differ from that in adult mice ($P = 0.085$ and 0.154, respectively). That is, the variability of the relative contribution of I_K and I_{Cl} to the outward currents found in the adult (rightmost plot in Fig. 1*B*) was achieved during the second postnatal week.

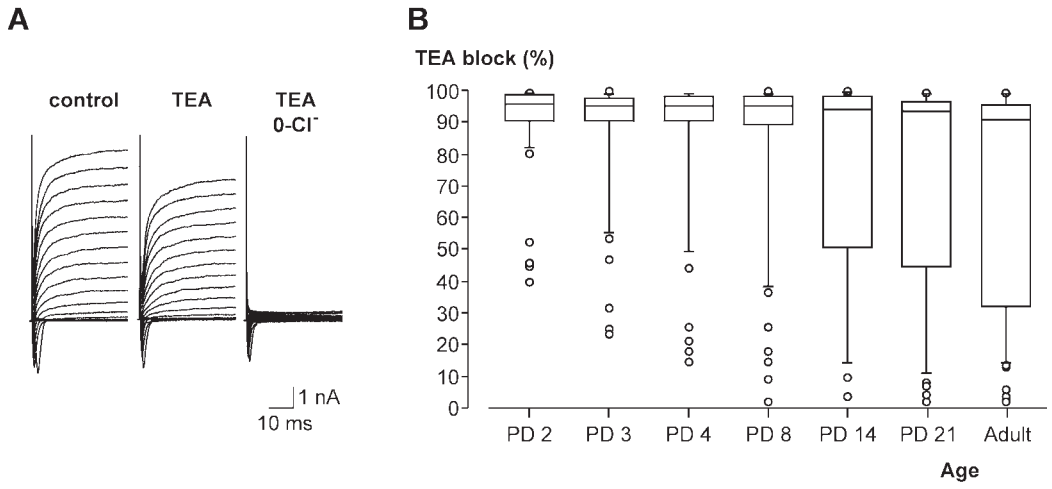

Figure 1 Contribution of voltage-gated K^+ and Cl^- currents (I_K and I_{Cl}, respectively) to the outward current in Na/OUT cells from mouse vallate papilla. **(A)** Membrane currents were elicited by a series of depolarizing pulses between −74 and +106 mV, in 10 mV increments, from a holding potential of −84 mV. Outward currents (upward deflections in the current records) in regular Tyrode solution (control) were partially abolished by the application of 20 mM TEA, indicating that this taste cell possessed I_K. The TEA-insensitive currents remaining during TEA application was totally abolished by removal of chloride ions from the bath solution (TEA 0–Cl^-). This was consistent with the presence of I_{Cl} in addition to I_K. Downward deflections in the current records: voltage-gated Na^+ currents. Patch pipette solution: K gluconate. **(B)** Box plot for the effect of 20 mM TEA on voltage-gated outward currents in Na/OUT cells during postnatal development. Inhibition by TEA was evaluated on currents elicited by depolarizing the membrane to +46 mV from a holding potential of −84 mV. In young animals (PD 2–8), the majority of Na/OUT cells expressed almost exclusively voltage-gated K^+ currents (I_K), as indicated by the position of the outlined box in the top of the graph. Cells expressing almost exclusively I_{Cl} (small TEA effect) were lacking at the early postnatal stages (PD 2–4) and appeared at PD 8. n = 49/49/49/58/34/44/63. PD, postnatal day.

Electrophysiological subtypes of Na/OUT cells during postnatal development

As shown by the variability of the TEA effect among Na/OUT cells, outward currents were composed of a varying combination of I_K and I_{Cl}. Interestingly, the box plots indicated that during development there was a gradual appearance of cells endowed with I_{Cl} (small TEA effect: see smaller extremes shown by lower circles in Fig. 1*B*). That is, these cells acquired more I_{Cl} progressively during development. In addition, the location of the median in the uppermost part of the box plots suggested that throughout the development there was a group of Na/OUT cells exhibiting only I_K. On the basis of these observations, we could empirically identified three main functional subtypes of Na/OUT cells, as follows: (i) cells possessing almost exclusively I_K (hereinafter, 'K cells'), in which TEA blocked >90% of the outward current; (ii) cells possessing both I_K and I_{Cl} (hereinafter, 'K+Cl cells'), in which TEA block was between 10 and 90%; and (iii) cells expressing almost exclusively I_{Cl} (hereinafter, 'Cl cells'), in which TEA block was <10%. As shown in Figure 2, K cells were the most numerous at all ages. K+Cl cells were also present throughout the development, although at a lower percentage. In contrast, Cl cells were absent during the early development, and appeared at PD 8 in our analysis.

The adopted classification allowed us to evaluate in detail the biophysical properties of the outward currents in functionally distinct Na/OUT cells during postnatal development. We recall, at this point, that results of a previous research by us demonstrated that in the whole population of these cells the average amplitude of outward currents increased with age (Bigiani *et al.*, 2002). Consistently, we found that a gradual increase in current amplitude occurred in both K cells and in K+Cl cells after birth (Fig. 3*A*,*B*). On the contrary, the amplitude of outward currents in Cl cells was fairly large at the beginning of their appearance (PD 8) and thereafter did not change much (Fig. 3*C*). As shown by the current-voltage relationships (*I*/*V* plots) in Figure 3, activation threshold for I_K and I_{Cl} did not change during development.

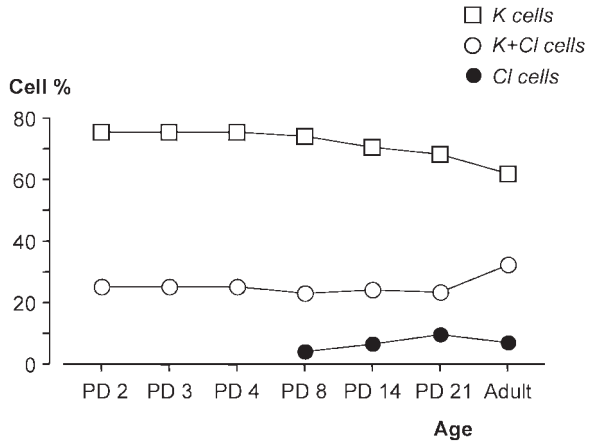

Figure 2 Electrophysiological subtypes of Na/OUT cells in the mouse vallate papilla during postnatal development. The percentage of each cell subtypes is shown. K cells and K+Cl cells occurred at all ages. In contrast, Cl cells were absent in the early developmental stages. Total number of tested cells for each age: 49/49/49/58/34/44/63, respectively, for seven developmental stages. PD, postnatal day.

We also measured the zero-current potential (V_0) in the three subtypes of Na/OUT cells during postnatal development. V_0, i.e. the pipette potential at which the overall ion fluxes through the membrane become zero, provides clues on the passive behaviour of the cell membrane under given ionic conditions. As shown in Figure 4, V_0 either in K cells and in K+Cl cells became more negative during development (at significantly lower levels in K+Cl cells). Interestingly, Cl cells showed the same, very negative V_0 of K+Cl cells since their appearance at PD 8.

Discussion

Taste cells in adult mammals are functionally heterogeneous as to the expression of ion channels. How the adult phenotypes are acquired during maturation processes that take place after birth is poorly understood. We have addressed this issue by studying the voltage-gated K^+ and Cl^- currents (I_K and I_{Cl}, respectively) occurring in a specific group of taste cells, the Na/OUT cells, in the mouse vallate papilla. These cells are thought to be sensory in function (Bigiani *et al.*, 2002). We have evaluated the relative contribution of I_K and I_{Cl} to the whole outward currents in Na/OUT cells at different postnatal ages and have found that this contribution changes with development. In the adult, there is a range of contributions of I_K and I_{Cl} to outward currents in Na/OUT cells. Some cells express almost exclusively I_K (K cells), some possess both I_K and I_{Cl} (K+Cl cells) and some almost exclusively I_{Cl} (Cl cells). In contrast, at the early stages of postnatal development (PD 2–4), only K cells and K+Cl cells could be detected in taste buds. The absence of Cl cells among patched cells at the early postnatal ages (PD 2–4) was not due to difficulties in accessing these cells in the taste buds with the patch pipettes. At this stage of development, mouse taste buds contain few cells and often isolated cells can be found after tissue dissociation (State and Bowden, 1974; Bigiani *et al.*, 2002). In addition, we patched a fairly large number of cells (147) between PD 2 and PD 4. At PD 21, taste buds are larger because they contained many cells. Nevertheless, we were able to detect five Cl cells out of 44 patched cells. The observations that Cl cells appeared at PD 8 and that the current amplitude did not change with development suggested that these cells could derive from other cell types, possibly the K+Cl cells. This was supported by the findings on V_0, which was similar in these two cell types (Fig. 4). It is therefore tempting to speculate that K+Cl cells and Cl cells might constitute a functional cell line in which the latter derive from some K+Cl cells. How this sprouting would be achieved during postnatal development remains unknown.

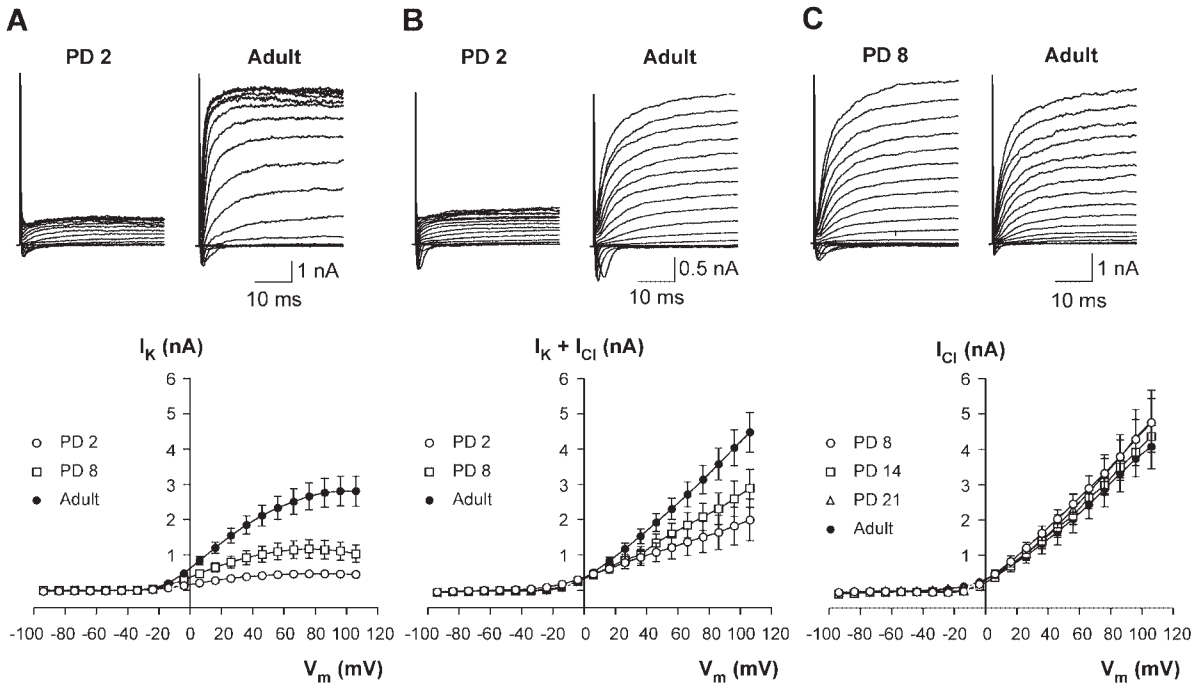

Figure 3 Voltage-gated outward currents in the three kinds of Na/OUT cells during postnatal development. Membrane currents were elicited by a series of depolarizing pulses between –74 and +106 mV, in 10 mV increments, from a holding potential of –84 mV. Outward currents (upward deflections in the current records) were carried by potassium ions and/or chloride ions. Downward deflections in the current records: voltage-gated Na^+ currents. The magnitude of outward currents markedly increased in K cells **(A)** and in K+Cl cells **(B)** during development, In contrast, outward currents were of similar magnitude in Cl cells regardless the age of the animal **(C)**. Current–voltage relationships of voltage-gated K^+ currents (I_K), K^+ and Cl^- currents ($I_K + I_{Cl}$) and Cl–currents (I_{Cl}) are shown at the bottom. Cells were pooled into three age groups (PD 2, PD 8, Adult) for K cells (A) and K+Cl cells (B), and into four groups (PD 8, PD 14, PD 21, Adult) for Cl cells (C). Amplitude values of the current for each membrane potential (V_m) were averaged within each group. Current amplitude was measured at the end of 40 ms pulses. Each point represents the mean ± SEM. n = 39/26/40 (A), n = 7/10/26 (B) and 3–5 (C). Note that at earlier developmental stages (PD 2–4), cells expressing only I_{Cl} were absent. PD, postnatal day; V_m, membrane potential.

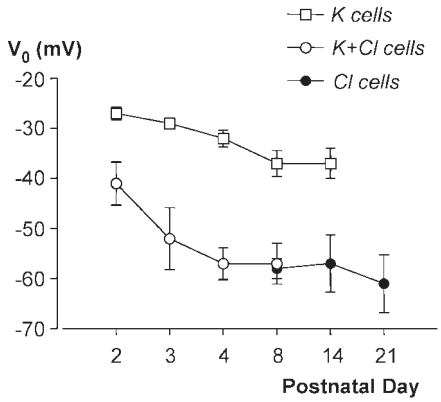

Figure 4 Zero-current potential (V_0) of Na/OUT cells during postnatal development. Cells were grouped into three functional subtypes and measurements of V_0 were averaged within each group and for any given postnatal day (open squares, n = 33/28/23/32/22; open circles, n = 7/6/5/6; filled circles, n = 3/4/7). V_0 becomes significantly more negative during development in K cells and in K+Cl cells. In contrast, cells expressing only I_{Cl} exhibit very negative V_0 (at the same level of the K+Cl cells) since their appearance.

One interesting aspect of the postnatal development of Na/OUT cell subtypes is the rate at which maturation processes occur. The large leap in functional heterogeneity takes place during the second week (compare the position of the boxes in Fig. 1*B*). During the first week, the distribution pattern does not change much and is characterized by the frequent occurrence of K cells among patched cells (boxes completely included in uppermost part in Fig. 1*B*). It seems like that the diversification process speeds up after PD 8. We think that this behaviour might be related to the morphogenetic processes underlying taste bud formation. In rodents, taste buds appear after birth and increase in size for the addition of new cells (State and Bowden, 1974; Hosley and Oakley, 1987; Kossel *et al.*, 1997; Bigiani *et al.*, 2002). As we demonstrated previously (Bigiani *et al.*, 2002), Na/OUT cells represent the main functional type in mouse vallate taste buds during early development. It is therefore reasonable to conceive that during the first week, taste buds are rapidly formed and increased in size owing to the appearance of the more common taste cells among Na/OUT cells, i.e. the K cells (Fig. 2). Incidentally, the number of taste buds in the mouse vallate papilla reaches the adult value by PD 8–10 (Cooper and Oakley, 1998; Oakley *et al.*, 1998), with an estimated time constant of ~4 days. This would be also consistent with our hypotheses that during the first week, developmental processes for Na/OUT cells might be more

focused on sizing up taste buds than on achieving functional diversification.

In taste cells of the adult rodents, I_K is modulated by several extracellular signals, including gustatory stimuli (Cummings *et al.*, 1996; Chen and Herness, 1997; Gilbertson *et al.*, 1997; Park *et al.*, 2003), leptin (Kawai *et al.*, 2000) and cholecystokinin (Herness *et al.*, 2002b). Findings by the Herness' group with rat taste cells indicate that adrenergic signalling enhances I_{Cl} (Herness and Sun, 1999) while inhibiting I_K (Herness *et al.*, 2002a). Caffeine, a bitter-tasting stimulus, inhibits I_K but has no effect on I_{Cl} (Zhao *et al.*, 2002). According to our data, we should expect a change in the 'picture' of peripheral taste modulation as the functional maturation of taste cells proceeds during postnatal development. This, of course, will have obvious repercussions on the operation of taste buds as input devices for controlling food selection and intake.

Histological studies have revealed the existence of different types of taste cells in adult mammals. A conservative description includes four main morphotypes: type I, type II and type III, which are elongated elements reaching the apex of the taste buds and therefore are thought to be, at least some of them, sensory cells; and basal cells, which may represent stem cells involved in taste bud turnover (Lindemann, 1996; Finger and Simon, 2000). How the functional subtypes of Na/OUT cells described in this study correlate with those morphological classes remains unknown. A nice study by Medler *et al.* (2003) has provided evidence for morpho-functional correlations in mouse taste cells. It was found that type I, II and III cells all displayed voltage-gated sodium currents, i.e. they were Na/OUT cells according to our functional nomenclature. Thus, findings by Medler and collaborators suggest that likely we analysed the electrophysiological properties of three distinct morphotypes, at least in the adult. It is not known, however, whether these morphological groups occur at all stages of the postnatal development.

In adult rodents, taste cells turnover in ~10–14 days (Beidler and Smallman, 1965; Farbman, 1980). An obvious question is then: do the changes of I_K and I_{Cl} during postnatal development reflect a mechanism that will operate also in adult animals? We noticed that the outward currents of low amplitude could be recorded in both K cells and K+Cl cells throughout the postnatal development (data not shown; see also Bigiani *et al.*, 2002). This observation was indicative of the appearance of new, immature taste cells throughout all ages and during cell turnover. In addition, once established during development, the electrophysiological heterogeneity is maintained in the adult (Fig. 1*B*). Thus, postnatal development and cell turnover are likely strictly related. It is tempting to speculate that changes in the functional expression of I_K and I_{Cl} in developing cells might represent the process that will take place during turnover in the adult. In this regard, our findings are consistent with electrophysiological data on taste cell turnover in *Necturus maculosus*, in which the maturation process involved, among other things, an increase in the magnitude of voltage-gated currents (Mackay-Sim *et al.*, 1996).

Acknowledgements

V.G. and F.F. contributed equally to the work. We thank Mrs Deanna Vecchi and Mr Giuseppe Nespoli for their excellent technical assistance. This study was supported in part by MIUR (Cofin 2000 to A.B.).

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