Early Dietary Sodium Restriction Disrupts the Peripheral Anatomical Development of the Gustatory System

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ABSTRACT: **Dietary sodium restriction has profound effects on the development of peripheral taste function and central taste system anatomy. This study examined whether early dietary sodium restriction also affects innervation of taste buds. The number of geniculate ganglion cells that innervate single fungiform taste buds were quantified for the midregion of the tongue in two groups of rats: those fed either a low-sodium diet and those fed a sodium replete diet (control rats) from early prenatal development through adulthood. The same mean number of ganglion cells in developmentally sodium-restricted and control adult rats innervated taste buds on the midregion of the tongue. However, the characteristic relationship of the larger the taste bud, the more neurons that innervate it did not develop in sodium-restricted rats. The failure to form such a relationship in experimental rats was likely due to a substantially smaller mean taste bud volume than controls and probably not to changes in innervation. Further experiments demonstrated that the altered association between number of innervating neurons and taste bud size in restricted rats was reversible. Feeding developmentally sodium-restricted rats a sodium replete diet at adulthood resulted in an increase in taste bud size. Accordingly, the high correlation between taste bud volume and innervation was established in sodium-replete rats. Findings from the current study reveal that early dietary manipulations influence neuron–target interactions; however, the effects of dietary sodium restriction on peripheral gustatory anatomy can be completely restored, even in adult animals.**

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Sensory systems undergo considerable modification during specific periods of development. It is during these developmental sensitive periods that the neural apparatus can be easily modified by environmental manipulations (Aslin, 1981). Numerous studies have examined the consequences of environmental manipulations during development with the ultimate goal of understanding mechanisms of normal development (e.g., Brunjes, 1994; Hubel and Weisel, 1970; Knudsen and Brainard, 1991; Renehan et al., 1989). That is, the goal of much of this work is to learn about normal development through complementary studies that use early environmental manipulations.

Although there has been relatively little work examining the role of environmental factors on the developing gustatory system, some important insights into taste system organization have been gained by the use of dietary sodium restriction. Restriction of maternal dietary sodium beginning on or before 8 days postconception and continued in offspring throughout

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development results in a 60% reduction of neurophysiological responses to sodium salts in the offspring's chorda tympani nerve. In contrast, taste responses to nonsodium salts and nonsalt stimuli are unaffected. The selective decrease in sodium salt-elicited responses is due to an apparent lack of functional amiloride-sensitive sodium channels in the apical domain of taste receptor cells (Hill, 1987; Hill et al., 1986; Hill and Przekop, 1988; Ye et al., 1993). Thus, adult rats that have been developmentally restricted of dietary sodium do not have functional amiloride-sensitive sodium channels and therefore have not developed normal sodium responses in the chorda tympani.

Although these initial dietary effects on taste system function are dramatic, they are reversible. Chorda tympani responses in restricted rats fed an NaCl-replete diet anytime during their life recover to control levels within 15 days (Przekop et al., 1990). This increase in sodium sensitivity is due to an increase in functional sodium channels (Hill, 1987). Thus, when rats are restricted of dietary sodium pre- and postnatally, the sodium taste system does not become functional. However, if restricted rats are returned to a sodium-replete diet in adulthood, normal sodium taste sensitivity occurs.

Environmental alterations have even more prominent effects on central gustatory system (King and Hill, 1993; Vogt and Hill, 1993). Dietary sodium restriction during pre- and postnatal development produces both an enlarged and an abnormally distributed chorda tympani terminal field in the nucleus of the solitary tract (NTS) (King and Hill, 1991). Recent findings have added information about the period during which this dietary manipulation has an effect on central gustatory organization. A brief, early, prenatal period of sodium restriction from postconception day 3 to 12 is sufficient to produce a permanent alteration in the chorda tympani field (Krimm and Hill, 1997). Thus, a prominent presynaptic morphological alteration at the first central gustatory relay occurs as a result of dietary manipulations during a limited time when chorda tympani neurons are born (Altman and Bayer, 1982) and long before sodium sensitivity develops normally (Hill and Almli, 1980; Hill et al., 1982; Sollars and Bernstein, 1994).

In addition to the central processes that form the terminal field in the NTS, geniculate ganglion neurons extend processes to the periphery via the chorda tympani nerve to innervate lingual taste buds in fungiform papillae. Therefore, it is possible that the same factors that determine central terminal field development also affect peripheral innervation patterns. Peripheral innervation patterns have been examined by determining the number of geniculate ganglion neurons that innervate a single taste bud (Krimm and Hill, 1998). In normal adult rats, there is a highly positive correlation ($r = 0.91$) between the number of geniculate ganglion cells that innervate a particular taste bud and the size of the taste bud. Thus, taste bud size is an accurate predictor of the number of ganglion cells that innervate it. This relationship is important because it provides the standard to which innervation in rats that are experimentally manipulated can be compared.

Since developmental sodium-restriction results in substantial changes in peripheral physiology and central morphology of the taste system, the current study was designed to determine whether sodium restriction also has an effect on the development of peripheral innervation patterns in the rat taste system. Specifically, does the adult relationship between taste bud size and number of innervating chorda tympani neurons develop under the conditions of developmental dietary sodium restriction? Alternatively, do the peripheral processes of the chorda tympani nerve in sodium-restricted rats show morphological changes resulting in a greater number of innervating ganglion cells? Finally, does feeding developmentally sodium-restricted rats a sodium replete diet in adulthood exaggerate any observed effects of sodium restriction, as evident in central terminal fields?

MATERIALS AND METHODS

Experimental Procedure and Design

Single fungiform papillae located in the midregion of the tongue were labeled with a fluorescent tracer in adult rats sodium-restricted throughout development (sodium-restricted rats; $n = 17$ papillae in 13 rats). In addition, single papillae on the midregion of the tongue were labeled with a fluorescent tracer in rats that were sodium restricted throughout development and repleted in adulthood (sodium-replete rats; $n = 13$ papillae in eight rats). Data from these groups were compared to those of normal control animals ($n = 16$ papillae in 15 rats). Since geniculate ganglion cells only innervate taste buds on the ipsilateral side of the tongue (Krimm and Hill, 1998), papillae on both sides of the tongue were examined independently. This allowed us to label single papilla on each side of the tongue with the same fluorescent tracer and multiple papillae on the same side of the tongue with different tracers.

Dietary Restriction Procedures

Sodium restriction during early development was accomplished by feeding pregnant rats (Harlan Sprague-Dawley; Indianapolis, IN) a sodium-deficient diet consisting of 0.03% NaCl (ICN-Nutritional Biochemicals) from 3 days postconception until the time of weaning (21 days postna-

tal). Pups were weaned to the sodium-deficient diet and remained on this diet throughout the experiment. Sodium-replete rats were fed the sodium-restricted diet until they were aged 45 days postnatal and were then fed a sodium-replete diet (1.0% NaCl). The age of sodium repletion (45 days) is past the age when the developmental relationship between taste bud size and innervating ganglion cells is complete (Krimm and Hill, 1998). The sodium-replete rats remained on the sodium-replete diet a minimum of 60 days before individual papillae were injected. Control rats were maintained on a sodium-replete diet (1.0% NaCl) throughout life.

Labeling Single Fungiform Papillae

The method for labeling single fungiform taste buds was as detailed by Krimm and Hill (1998). Briefly, adult rats were anesthetized with sodium pentobarbital [50mg/kg, intraperitoneally (i.p.)] or sodium Brevital (60 mg/kg, i.p.) and placed on a water-circulating heating pad to maintain body temperature at 36°C. The dorsal, anterior half of the tongue was exposed from the mouth by gently pulling on the ventral tongue. The tongue was stabilized by pressing the ventral surface to a glass slide covered with putty, and fungiform papillae were visualized with the aid of a 0.5% solution of Methylene blue (Fischer Scientific) painted on the tongue surface. Using a micromanipulator and surgical microscope, a glass pipette (150 μm in diameter) was placed over single papillae located in the midtongue region without penetrating the epithelium, yet firm enough to create an electrical seal [Figs. 1(A) and 2]. A small wire (0.3 mm in diameter), inserted into the ventral tongue, served as the reference electrode. By applying a square, anodal pulse (Grass Electronics; 0.5–1.0-mA positive current, 4 s on/4 s off for 5–10 min), one of two fluorescent dyes were iontophoresed into papillae: True Blue chloride (Molecular Probes; 2% in distilled water) [Fig. 1(A)] or Fluoro-Gold (Fluorochrome Inc.; 2% in distilled water). The presence of an electrical seal and passage of current was monitored with an ammeter.

Histological Procedures

Rats were sacrificed with a lethal dose of sodium pentobarbital 4 days after application of the label and perfused intracardially with physiological saline, followed with 4% paraformaldehyde (pH 6.5 followed by pH 9.5). Tongues and geniculate ganglia were removed and placed in 30% sucrose overnight. The distances between labeled papillae and the intermolar eminence and between papillae and the midline were measured to determine the location of the papillae on the tongue surface. Thus, a map was made of the locations of sampled papillae on the tongue surface for regional examination of innervation patterns. Each block of tissue was positioned in a plastic embedding mold, covered with Histotech, and frozen at -70°C until sectioning. Serial 10-μm sections of geniculate ganglia and 20-μm sections of tongue were obtained with a cryostat, thaw-mounted on gelatin-coated glass slides, cleared with xylenes, and coverslipped with DPX. After examination of the amount of fluorescent label within papillae [Fig. 1(A)], coverslips were removed and slides containing tongue sections were stained with hematoxylin and eosin [Fig. 1(C)].

Data Analysis

All tissue was examined under a microscope with an epifluorescent illuminator [Fig. 1(A,B)]. Successful labels were ones in which the label filled the entire taste bud but were contained within the dorsal half of the papilla [Fig. 1(A)]. Only ganglia from successful labels were analyzed.

Analyses of ganglia were accomplished by serially reconstructing each ganglia with a computer microscope system (Neurolucida; Microbrightfield). Digital information of *X*, *Y*, and *Z* coordinates were fed on-line to a computer as tracings were made of the borders of the ganglia and of labeled cells. Ganglion cells were counted without experimenter knowledge of taste bud size.

To measure taste bud volumes, the perimeter of the taste bud in serial sections were outlined and corresponding area was computed by an Olympus Cue-2 image analysis system. The areas were multiplied by section thickness and summed to derive an estimate of the total taste bud volume. To achieve consistency with other reports, the borders of taste buds were drawn such that peripheral cells of the taste bud were included in the measurement (Whitehead et al., 1985; Krimm and Hill, 1998) [Fig. 1(C)]. Taste buds were measured without experimenter knowledge of ganglion cell number. In addition to the labeled taste buds, a sample of 50 unlabeled taste buds for the midregion of the tongue in animals comprising each of the three experimental groups was stained with hematoxylin and eosin, subsequently measured, and analyzed separately from labeled taste buds. This was done to determine whether labeled papillae were representative of the total population of taste buds. It also allowed a more reliable examination of differences in taste bud size among experimental groups.

Statistics

Taste bud volumes of labeled taste buds were compared to the additional 50 taste bud volume measured from the same group with a *t* test. Analysis of variance (ANOVA) was used to compare mean taste bud size and mean number of ganglion cells among groups. Following a significant ANOVA, multiple comparisons were made using the Bonferroni *t* test. Pearson product-moment correlations were used to analyze the relationship between taste bud size and number of innervating ganglion cells. The α level was set at $p < .05$.

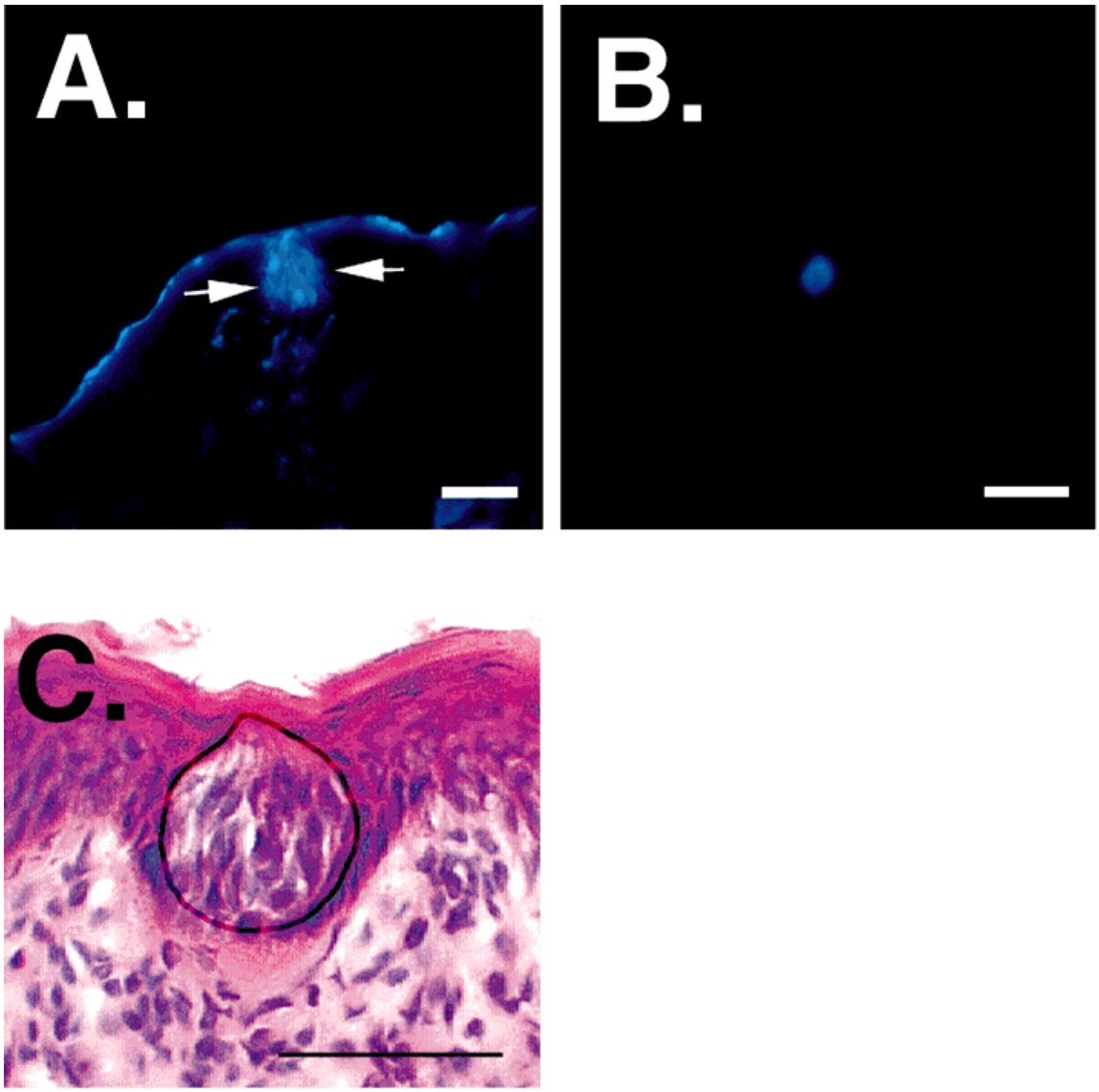

Figure 1 Photomicrographs of a taste bud labeled with True Blue [(A), between arrows] and a corresponding labeled geniculate ganglion cell (B). A hematoxylin and eosin–stained taste bud used for volume measurements is shown in (C). The line overlaying the taste bud denotes the borders used in obtaining area measurements. All tissue are from control animals. Scale bars = 50 μm.

RESULTS

Sample Taste Buds Are Representative of the Population

There were no differences between the labeled taste bud volumes and the 50 unlabeled taste bud volumes measured from the mid-region of the tongue for any group (Fig. 2) (normal adults, $p = .86$; sodium-restricted, $p = .19$; sodium-replete, $p = .39$). Thus, taste bud volumes for the labeled midregion papillae were representative of the taste bud volumes.

Numbers of Innervating Ganglion Cells following Developmental Sodium Restriction

As observed previously for normal adult rats (Krimm & Hill, 1998), there was substantial variation in the number of ganglion cells that innervated individual taste buds on the tongue midregion for both sodium-restricted (range, 3–14) and sodium-replete rats (range, 4–14). The mean number of ganglion cells innervating fungiform papillae in the midregion of the rat tongue was the same among experimental groups

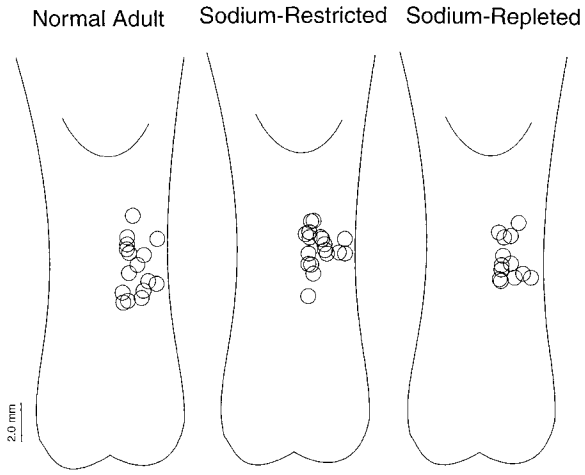

Figure 2 Locations of the fungiform papillae (circles) that were labeled in normal adult, sodium-restricted and sodium-replete rats. Injections were made in approximately the same region for all three groups.

[$F(2, 44) = 0.85$, $p = .43$]. Thus, there was no effect of developmental dietary sodium restriction on the average number of ganglion cells innervating single taste buds.

Taste Bud Volumes following Developmental Sodium Restriction

Although taste buds in all three experimental groups were innervated by the same mean number of ganglion cells, taste bud volumes differed significantly between groups (Fig. 3) [$F(2, 139) = 14.8$, $p < .0001$]. Taste bud volumes in adult rats that were sodium restricted throughout development were substantially smaller than in normal adults ($t = 5.56$, df $= 98$, $p < .000001$). Thus, developmental sodium restriction affected taste bud growth. In contrast, taste bud volumes of rats that were developmentally sodium restricted and then sodium repleted in adulthood were not different from normal adult rats ($p = .17$). Therefore, taste buds increased to their normal size when sodium-restricted rats were placed on a sodium-replete diet for 60 days.

Relationship between Taste Bud Size and Innervation in Developmentally Sodium Restricted Rats

In normal control rats, the number of ganglion cells innervating a fungiform papilla predicts the volume of the corresponding taste bud (Krimm and Hill, 1998) (Fig. 4) ($r = 0.90$, $p = .0001$). However, there was no correlation between taste bud volume and number of innervating ganglion cells in sodium restricted rats (Fig. 4) ($p > .43$). Thus, along with decreased taste bud volume, dietary sodium restriction prevented the development of the relationship between taste bud size and number of innervating ganglion cells. It appears, however, that this effect was not permanent. Sodium-replete rats had a significant correlation between taste bud volume and number of innervating ganglion cells (Fig. 4) ($r = 0.71$, $p = .007$). When plotted on the regression line for control rats, these data appear to fit the regression line for the relationship between taste bud size and number of labeled ganglion cells in normal adult rats (Fig. 4). In fact, 12 of the 13 data points for the sodium-replete group were within the 95% confidence intervals calculated from the data in normal control rats. Therefore, while dietary sodium restriction prevents the formation of the relationship between taste bud size and innervation during development, feeding animals a sodium replete diet allowed this relationship to recover even in adulthood.

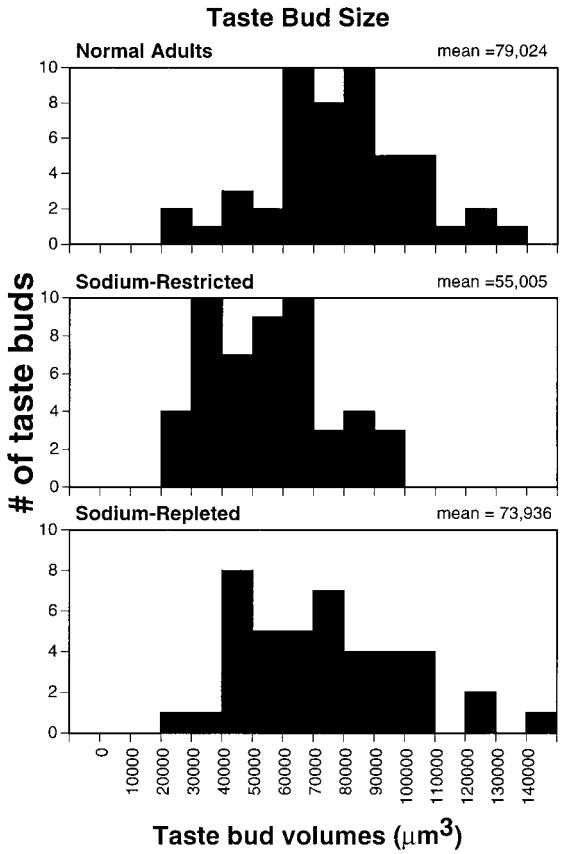

Figure 3 Histograms showing the number of taste buds of different volumes for normal adult (top), sodium-restricted rats (middle), and sodium-repleted rats (bottom). Taste buds in sodium-restricted rats are smaller than those of normal adult rats and in sodium-repleted rats.

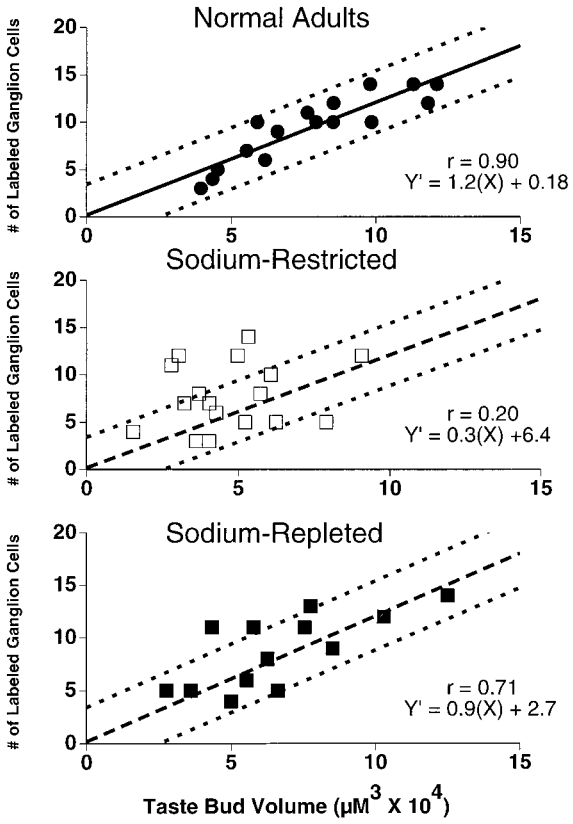

Figure 4 Number of labeled geniculate ganglion cells plotted against taste bud volumes for taste buds sampled from the tongue midregion in normal adults (top). The 95% confidence intervals for the regression line are displayed with dotted lines. The number of labeled geniculate ganglion cells is plotted against taste bud volumes for taste buds sampled from the tongue midregion in sodium-restricted (middle) and sodium-replete rats (bottom). The regression lines in the top and bottom panels are replotted from (A) and are shown as a dashed line. There is no relationship between taste bud volume and number of labeled ganglion cells in sodium-restricted rats. However, there is a positive correlation between taste bud volume and number of labeled ganglion cells in sodium-replete rats. The correlation coefficients and equations for regression lines for each group are shown in the bottom right of the respective panels.

Regional Differences in Innervation

For the purposes of examining regional differences in taste bud size and amount of innervation, medial and lateral portions of the tongue were differentiated and the number of ganglion cells innervating medial and lateral taste buds were analyzed. Papillae located within 1.2 mm of the midline were considered medial papillae and those more lateral than 1.2 mm were considered to be lateral papillae (Krimm and Hill, 1998).

Regional differences have been demonstrated in innervation across the tongue in normal adult rats (Krimm and Hill, 1998). Specifically, taste buds near the midline are innervated by more geniculate ganglion cells than lateral taste buds. Accordingly, regional differences were examined in rats that were sodium-restricted during development. Medial taste buds in both sodium-restricted rats and sodium-replete rats were innervated by more ganglion cells than lateral taste buds ($t = 2.35$, df $= 16$, $p < .02$; $t = 3.58$, df $= 11$, $p < .003$). Thus, the same regional differences in ganglion cell innervation that has been observed for normal adult rats also occurred in rats restricted of sodium during development.

In addition to greater numbers of innervating ganglion cells, medial taste buds are larger than lateral taste buds in normal adult rats (Krimm and Hill, 1998). In sodium-restricted rats, there was no difference in taste bud volume between medial and lateral papillae ($p > .15$). However, once rats were fed a normal sodium-replete diet at 45 days of age, medial to lateral differences in taste bud size emerged ($t = 1.88$, df $= 11$, $p < .04$). Thus, although regional differences in numbers of innervating ganglion cells occurred under conditions of sodium restriction, regional differences in taste bud size did not develop in developmentally sodium-restricted rats. These regional differences in taste bud size can be recovered once rats are fed a normal sodium-replete diet.

DISCUSSION

Findings from the current study reveal that the peripheral gustatory system is susceptible to environmental influences during development. Specifically, dietary sodium restriction disrupts both the development of taste buds and the relationship between taste buds and number of innervating neurons. Developmental sodium restriction retards the maturation of taste buds such that they are substantially smaller in rats restricted of sodium. By disrupting the development of taste bud volume, sodium restriction also disrupts the development of the relationship between taste bud size and innervation that is typically observed in normal rats. Thus, a very orderly relationship between morphology and innervation, which is present is normal adult rats, is disrupted by sodium restriction. Interestingly, there is not an effect of sodium restriction on the average number of innervating ganglion cells. Therefore, the effect of sodium restriction on the development of the relationship between taste bud size and innervation appears to be due primarily to a reduction in taste bud volume. Feeding sodium-re-

stricted rats a sodium-replete diet in adulthood results in recovery of taste buds to a normal volume. Accordingly, the relationship between taste bud volume and number of innervating ganglion cells becomes established.

Comparison with Normal Development

The relationship between taste bud size and number of innervating chorda tympani neurons seen in adult rats develops over a long postnatal period (Krimm and Hill, 1998). Specifically, in normal rats, taste buds continue to increase in volume through postnatal day 40 (Krimm and Hill, 1998). Therefore, much like sodium-restricted rats, the correlation between taste bud size and number of innervating cells in postnatal rats does not appear until taste bud size reaches maturity. Interestingly, when the data for sodium restricted animals and for normal developing rats are compared, sodium restricted rats most closely resemble 20-day-old rats for taste bud size and the relationship between taste bud size and number of innervating ganglion cells. Therefore, it appears that sodium restriction causes the development of both taste bud size and the relationship between taste bud size and number of innervating ganglion cells to be "frozen" at an immature state.

Regional comparisons across the tongue surface also demonstrate the similarity of sodium-restricted and developing rats. Adult sodium-restricted rats are similar to developing rats in that more ganglion cells innervate medial taste buds than lateral taste buds. Not surprisingly, sodium-replete rats are similar to adult rats in that a medial to lateral difference is present in both taste bud size and numbers of innervating ganglion cells. Development of regional differences in taste buds size is halted by sodium restriction, but can develop in adult rats once they are returned to a sodium-replete diet. These data also imply that the mature complement of neurons innervating a taste bud is established early in development in both sodium restricted and replete rats, and that it is the taste bud size that grows to match the innervation pattern. In the case of normal development, taste bud size increases with age. In the case of developmental sodium restriction, taste bud size increases when the rats are fed a sodium-replete diet.

Comparison with Central Terminal Field Effects

One purpose of this study was to determine whether sodium restriction affects peripheral innervation patterns of chorda tympani neurons as well as their central terminal fields. As noted above, there appears to be relatively little influence on the innervation pattern of single fungiform taste buds. Thus, unlike the central processes of the chorda tympani nerve, where there is a substantial reorganization of the terminal field in developmentally sodium-restricted rats, the number of peripheral processes that innervate single taste buds seem unaffected.

In addition to having a different locus of effect, central and peripheral taste anatomy differ in their ability to recover from developmental sodium restriction. Developmental sodium restriction is required for only a brief period during embryonic development for permanent changes to occur in the central chorda tympani terminal field (Krimm and Hill, 1997). The effects of sodium restriction on central terminal field development cannot be recovered in rats placed on a normal sodium-replete diet, regardless of how long exposure to the sodium-replete diet is maintained. In contrast, the peripheral anatomical effects of sodium restriction are recovered in sodium-restricted rats exposed to a normal sodium replete diet in adulthood. Given the differences between the ability of the central and peripheral taste systems to recover, it seems likely that different mechanisms account for the anatomical effects of sodium restriction in these two areas.

Comparison with Peripheral Physiology

Sodium restriction during early embryonic development results in a dramatic decrease in neurophysiological responses to sodium salts in the chorda tympani nerve, owing to an absence of functional amiloride-sensitive sodium channels (Hill, 1987; Hill et al., 1986; Hill and Przekop, 1988). Unlike the central anatomical effects of sodium restriction, the peripheral physiological effects of sodium restriction on taste physiology can be completely recovered in adulthood (Przekop et al., 1990: Stewart and Hill, 1996). We showed that the reduction in taste bud size that results from developmental sodium-restriction can also be recovered in adult animals. It appears that the peripheral taste system retains the ability to recover from environmental manipulations throughout the animal's life. However, we do not suggest that functional changes and the changes in taste bud size with the dietary manipulation are directly related. A decrease in functional amiloride channels is not necessarily directly related to the change in taste bud volume. Such a functional and anatomical link will be established by linking the cellular mechanism for the volume changes with corresponding changes in functional amiloride channels.

Possible Explanations for Sodium Restriction Effect on Taste Bud Size

Sodium restriction may cause a reduction in taste bud size by reducing cell volume and/or reducing the number of taste cells. The effects on tissue growth and protein synthesis by dietary sodium restriction greatly exceed its effects on fluid volume (see Haycock, 1993, for review). Sodium may act as a growth factor by directly stimulating cell proliferation through the sodium-hydrogen antiporter (Mendoza, 1987; Pouyssegur, 1987). It is also possible that sodium restriction disrupts the production of some other factor or factors that lead to a reduction in taste bud size. Therefore, sodium would have an indirect effect on taste bud size. In fact, there is evidence that systemic factors may mediate the effects of sodium restriction on the peripheral physiological and central anatomical development of the taste system (Stewart and Hill, 1996; Krimm and Hill, 1997). Therefore, taste bud size could be influenced indirectly by sodium via another blood-borne factor. Since sodium restriction disrupts normal taste bud growth, determining which factor(s) mediate this effect could contribute substantially to our understanding of taste bud development. More basic information about whether changes in taste bud size results from increased taste bud cell numbers and/or increased size per taste cell would point to the specific factor(s). Moreover, questions related to the number of synapses on the cells and how they are modified by dietary manipulations are important for a thorough understanding of the dynamics of the developing gustatory system.

In summary, these results show that peripheral gustatory anatomy is susceptible to environmental manipulations during development. Specifically, sodium restriction "freezes" taste bud development at an immature state, which results in a disruption of the correlation between number of chorda tympani neurons and taste bud size. Similar to the peripheral physiological effects, but dissimilar to the central anatomical effects of sodium restriction, the peripheral anatomical effects recover in rats fed a sodium-replete diet. This demonstrates that the relationship between taste bud size and number of innervating ganglion cells can become established in adult rats.

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