

Gustatory Terminal Field Organization and Developmental Plasticity in the Nucleus of the Solitary Tract Revealed Through Triple-Fluorescence Labeling

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ABSTRACT

Early dietary sodium restriction has profound influences on the organization of the gustatory brainstem. However, the anatomical relationships among multiple gustatory nerve inputs have not been examined. Through the use of triple-fluorescence labeling and confocal laser microscopy, terminal fields of the greater superficial petrosal (GSP), chorda tympani (CT), and glossopharyngeal (IX) nerves were visualized concurrently in the nucleus of the solitary tract (NTS) of developmentally sodium-restricted and control rats. Dietary sodium restriction during pre- and postnatal development resulted in a twofold increase in the volume of both the CT and the IX nerve terminal fields but did not affect the volume of the GSP terminal field. In controls, these nerve terminal fields overlapped considerably. The dietary manipulation significantly increased the overlapping zones among terminal fields, resulting in an extension of CT and IX fields past their normal boundaries. The differences in terminal field volumes were exaggerated when expressed relative to the respective NTS volumes. Furthermore, increased terminal field volumes could not be attributed to an increase in the number of afferents because ganglion cell counts did not differ between groups. Taken together, selective increases in terminal field volume and ensuing overlap among terminal fields suggest an increased convergence of these gustatory nerve terminals onto neurons in the NTS. The genesis of such convergence is likely related to disruption of cellular and molecular mechanisms during the development of individual terminal fields, the consequences of which have implications for corresponding functional and behavioral alterations. *J. Comp. Neurol.* 497:658–669, 2006. © 2006 Wiley-Liss, Inc.

Indexing terms: brainstem nuclei; primary afferents; taste; confocal laser microscopy; sodium; diet

Neural plasticity has been widely studied as a way not only to examine the brain's compensatory strategies but also to characterize normal developmental processes. Some of the most dramatic and widely cited examples of neural plasticity involve higher order cortical processing and associated circuitry in sensory systems (Hubel and Weisel, 1970; Henderson et al., 1992; Buonomano and Merzenich, 1998; Catalano and Shatz, 1998; Rauschecker, 1999; Fox et al., 2002; Katz and Crowley, 2002; Yan, 2003). However, unlike other systems, the gustatory system shows a remarkable amount of developmental plasticity at the first synaptic relay in the brainstem, in the nucleus of the solitary tract (NTS).

Much of the research on the developing gustatory NTS has focused on the unique plasticity of chorda tympani (CT) nerve terminations in response to early environmen-

tal manipulations (Lasiter and Kachele, 1990; King and Hill, 1991; Lasiter and Diaz, 1992; Lasiter, 1995; Krimm and Hill, 1997; Pittman and Contreras, 2002). In rats fed a low-sodium diet (0.03% NaCl) from at least E3 to E12,

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the dorsalmost portion of the CT field expands caudally, to more than twice the size of controls (King and Hill, 1991; Krimm and Hill, 1997). The morphological alterations in the CT terminal field are permanent in that the effects are not reversible by age or by sodium repletion. In fact, feeding a sodium-replete diet at adulthood to developmentally sodium-restricted rats exaggerates the effects (King and Hill, 1991). This phenomenon, brought about by dietary sodium manipulation, appears to be selective to the CT field and does not affect other gustatory nerve terminal fields [e.g., the glossopharyngeal (IX) and greater superficial petrosal (GSP) nerves, King and Hill, 1991; Sollars and Hill, 2000].

Although previous tract tracing studies of gustatory projections to the NTS have provided the foundation for an understanding of the gross organization of gustatory terminations (Contreras et al., 1982; Hamilton and Norgren, 1984; Lasiter et al., 1989; Lasiter, 1992), the detailed organization of multiple gustatory terminal fields has not been examined simultaneously in a single animal. Among the tract tracing studies previously published, at most two tracers have been used in an individual animal to identify terminal fields. Previous experiments have labeled the facial (VII) nerve (Lasiter et al., 1995), which is composed of both CT and GSP, or individual gustatory nerves were labeled on opposing sides of the animal (Hamilton and Norgren, 1984). Furthermore, these studies have not been performed in living animals or they used horseradish peroxidase, both of which compromise the fidelity of the terminal field label. Finally, terminal fields were observed and reconstructed by using transmitted light microscopy and not confocal laser microscopy. Therefore, it is difficult to precisely identify and measure the zones of overlapping fields.

To explore fully the plasticity of gustatory terminal fields in the brainstem, we developed a triple-fluorescence labeling technique that could be used with confocal laser microscopy. The present study used these techniques to uncover unexpected and dramatic relationships among three gustatory terminal fields in developmentally sodium-restricted and control rats.

MATERIALS AND METHODS

All animal procedures were carried out in accordance with NIH guidelines for humane handling of animals, and all protocols were approved by the Institutional Animal Care Committee at the University of Virginia.

Animals

Eighteen adult (50–60 days old), female Sprague-Dawley rats (Harlan, Indianapolis, Indiana) maintained on either a control ($n = 9$) or a sodium-restricted ($n = 9$) diet were used to visualize simultaneously the three gustatory terminal fields in the NTS. Sodium restriction was established by feeding pregnant rats a 0.03% NaCl diet (ICN Biomedicals, Aurora, OH) and distilled water ad libitum from 3 days postconception through weaning at 21 postnatal days. Once pups born to these mothers were weaned, they were maintained on the same dietary regimen through adulthood. Control rats were the offspring of dams maintained on the standard 1% NaCl rat chow and tap water ad libitum. Control pups were then weaned to the standard diet at 21 postnatal days.

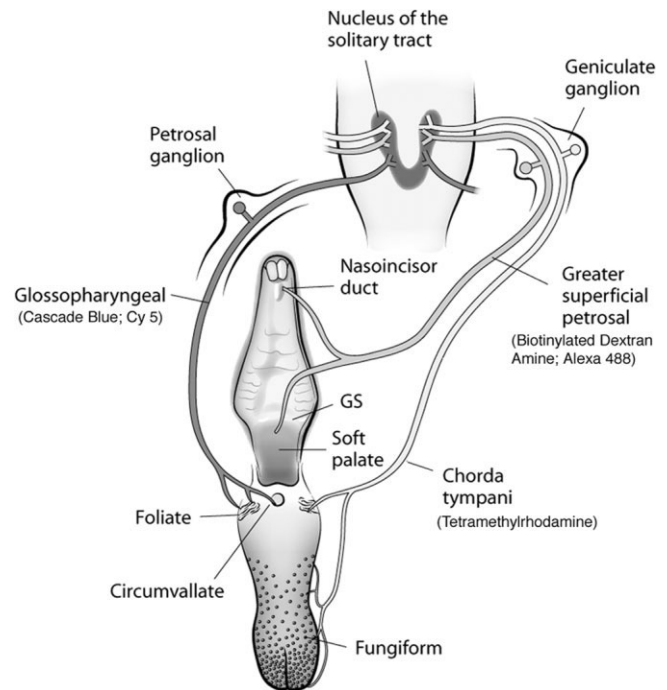


Fig. 1. Anatomical organization of the peripheral gustatory system and the first central synaptic relay in the nucleus of the solitary tract (NTS). The chorda tympani (CT) nerve innervates taste buds in fungiform and foliate papillae on the anterior tongue. The greater superficial petrosal (GSP) nerve innervates taste buds in the nasoincisor duct, the geschmacksstreifen (GS), and the soft palate. Both nerves make up the VIIth cranial nerve and have cell bodies in the geniculate ganglia. The glossopharyngeal (IX) nerve innervates taste buds in foliate and circumvallate papillae on the posterior tongue and has cell bodies in the petrosal ganglia. All three nerves terminate bilaterally in the NTS; the glossopharyngeal nerve is shown at left only for illustrative purposes. The fluorescent markers used to detect each terminal field are noted in parentheses. See Materials and Methods for a detailed description.

Triple-fluorescence anterograde labeling

To examine the arrangement of the gustatory terminal fields within the NTS, the CT, GSP, and IX nerves projecting to the right NTS were consecutively labeled with one of three anterograde neural tracers (Fig. 1). Rats were anesthetized with a 100 mg/kg injection of ketamine *i.m.* Supplementary injections were given as needed, and body temperature was maintained at 36°C with a water-circulating heating pad. Upon placement in a nontraumatic head holder (Erickson, 1966), a ventral approach, modified from Sollars and Hill (2000), was taken to expose the GSP and CT nerves in the right tympanic bulla. A longitudinal incision in the ventromedial portion of the neck was made, and the masseter muscle and the posterior belly of the digastric muscle were retracted. A small hole was made in the ventral aspect of the tympanic bulla to gain access to the CT and the GSP. First the GSP and then the CT nerve were cut and labeled near the geniculate ganglia. After a brief application (approximately 20 seconds) of dimethyl sulfoxide (DMSO), crystals of 3-kD biotinylated dextran amine (Molecular Probes, Eugene, OR) and 3-kD tetramethylrhodamine dextran amine (Molecular Probes) were carefully placed on the proximal cut

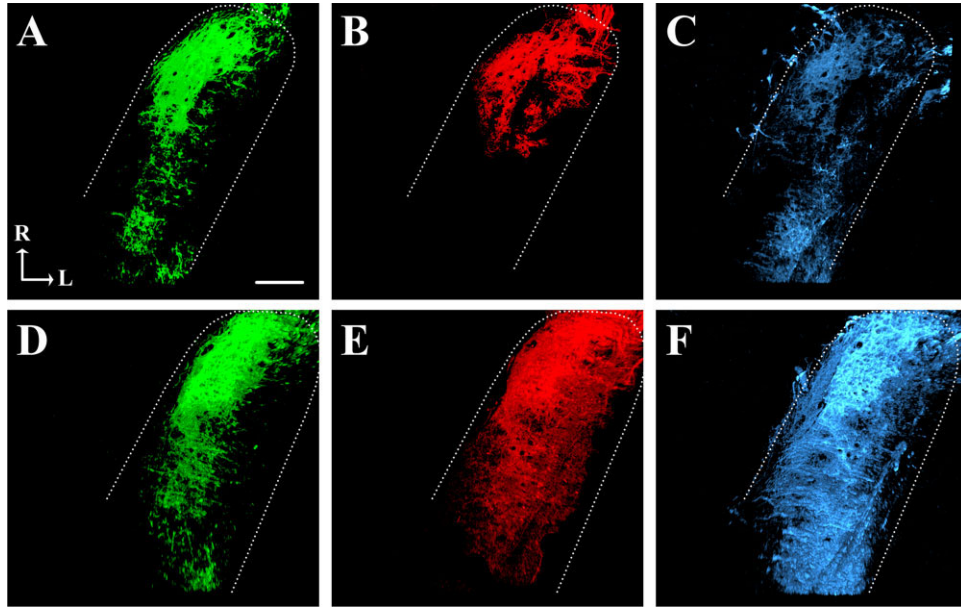


Fig. 2. Fluorescent photomicrographs of separate channels of the GSP (green: **A,D**), CT (red: **B,E**), and IX (blue: **C,F**) terminal fields in the dorsal zone of NTS (perimeter marked by dotted line) in control (**A–C**) and sodium-restricted (**D–F**) rats. Note the enlarged appearance of the

CT and IX terminal fields in sodium-restricted rats compared with controls. See Figure 3B,F for corresponding merged images of these separate channels. R, rostral; L, lateral. Scale bar = 100 μ m.

ends of the GSP and CT, respectively (Fig. 1). Petroleum jelly was applied over the cut ends of each nerve to hold dye crystals in place, and a piece of parafilm was placed over the opening created in the tympanic bulla. Next, the IX nerve was approached in the ventral side of the neck medial to the tympanic bulla. The nerve was cut and the proximal stump was teased onto a section of parafilm. Crystals of 3-kD Cascade blue dextran amine (Molecular Probes) were applied to the proximal cut end after a short application of DMSO (Fig. 1). Petroleum jelly and a layer of parafilm were placed on top of the nerve to secure dye placement. Care was taken to ensure that other nerves in the vicinity (e.g., superior laryngeal nerve-branch of the vagus) were not inadvertently cut and labeled. The incision was then sutured, and the rat remained on the heating pad until it recovered from the anesthetic. The optimal time for transport of the three neural tracers was determined through pilot data to be approximately 24 hours. Rats were killed after 24 hours and were perfused transcardially with Krebs solution (pH 7.3) followed by 8% paraformaldehyde (pH 7.0).

Tissue preparation

Brains were removed and postfixed in 8% paraformaldehyde overnight. After removal of the cerebellum, the medulla was blocked and sectioned on a vibratome horizontally at 50 μ m through the entire NTS. This allowed visualization of the entire rostral-caudal and medial-lateral extent of the terminal fields (Davis, 1988; Whitehead, 1988; Lasiter et al., 1989). Tissue sections were collected in 0.1 mM phosphate-buffered saline (PBS; pH 7.4) and remained free floating at room temperature throughout the remainder of the tissue preparation. Sections were incubated for 1 hour in 0.2% Triton X in PBS with a mixture of streptavidin Alexa fluor 488 (Molecular

Probes) at 1:500 to visualize GSP terminals labeled with biotinylated dextran amine and rabbit anti-Cascade blue (Molecular Probes) at 1:500 ultimately to allow visualization of IX terminals labeled with Cascade blue (Fig. 1). Sections were rinsed in PBS and subsequently incubated for 30 minutes in 0.2% Triton X and goat anti-rabbit Cy5 (Jackson Immunoresearch, West Grove, PA) at 1:500 to visualize IX terminals (Fig. 1). Sections were rinsed again in PBS and stored at 4°C until imaged. These fluorescent dyes were selected specifically for their particular excitation/emission wavelengths to avoid crossover of visible fluorescence. That is, each fluorochrome used had a distinct enough range of excitation that did not overlap significantly with the emission curves of the other two dyes, allowing visualization of each field with a confocal laser microscope system.

Confocal microscopy and data collection

Terminal fields were imaged with an Olympus IX70 microscope fitted with a Fluoview 3.3 confocal laser scanning system (Olympus America, Melville, NY) equipped with an argon-ion laser (488 nm; used to visualize the GSP terminal field), a helium-neon laser (543 nm; used to visualize the CT terminal field), and a helium-neon laser (633 nm; used to visualize the IX terminal field; Fig. 1). Each 50- μ m section containing terminal field label was mounted between coverslips with a 9:1 mixture of glycerol and PBS, and optical images were captured every 3 μ m for a total of approximately 17 images per section. Initially, for each 50- μ m physical section, the GSP and CT fields were imaged with sequential passes of the blue (argon-ion laser) and green (helium-neon; 543 nm) lasers and collected as separate channels (Fig. 2A,B,D,E). Then, by using the same parameters set for the initial scan, the IX field was imaged with the red laser (helium-neon; 633 nm)

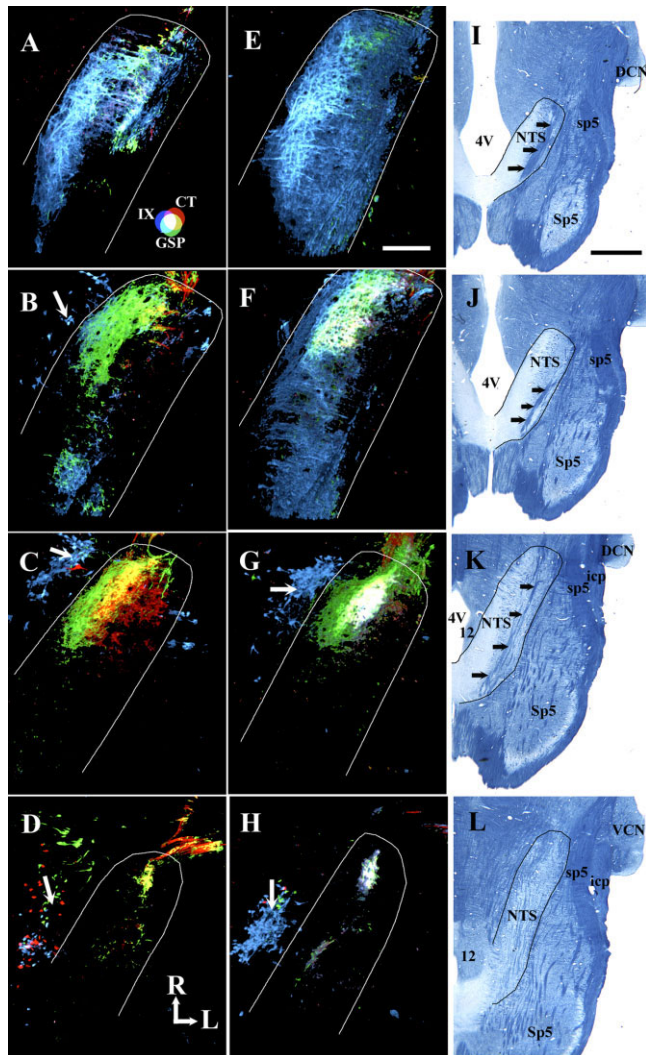


Fig. 3. Fluorescent photomicrographs comparing the triple-labeled terminal fields in horizontal sections of the NTS (approximate perimeter marked by white line) in a control (A–D) and a sodium-restricted (E–H) rat. The CT and IX terminal fields in the dorsal zone of restricted rats (E,F) are more expansive than control (A,B). Sections A and E are dorsal to B and F. In the intermediate zone, the appearance of terminal fields in sodium-restricted rats (G) is also different from control (C). Often, the IX terminal field in restricted rats extends into the ventral zone (H); however, this does not occur in controls (D). Retrogradely labeled salivatory nuclei are indicated by white arrows (B–D,G,H). I–L show myelin-stained tissue from a separate control rat illustrating the shape of the NTS and other brainstem structures at the respective dorsal to ventral level to the fluorescent images in the same row. The NTS is outlined in black, and black arrows point to the solitary tract. Refer to the color key in A to identify individual fields and overlap among different terminal fields. R, rostral; L, lateral. 4V, fourth ventricle; 12, hypoglossal nucleus; DCN, dorsal cochlear nucleus; icp, inferior cerebellar peduncle; NTS, nucleus of the solitary tract; Sp5, spinal trigeminal nucleus; sp5, spinal trigeminal tract; VCN, ventral cochlear nucleus. Scale bars = 200 μ m in E (applies to A–H); 1 mm in I (applies to I–L).

and saved as a third channel (Fig. 2C,F). Finally, by using FluoView, all three channels were merged to create a composite view (Fig. 3).

Terminal field volume and corresponding volumes of overlap among all three terminal fields and between individual terminal fields (Fig. 3) were quantified in NeuroLucida version 4.34 (MicroBrightField, Inc., Colchester, VT). Briefly, the perimeter of each labeled terminal field and the overlap among these fields were outlined, and the area was calculated for each 3- μ m optical section. Subsequently, the volume of each 50- μ m physical section was calculated by summing the areas of each optical section and multiplying by 3, because 3 μ m was the thickness between optical sections. Total terminal field volumes and volumes of overlap with other fields were determined by summing the volumes of each 50- μ m section. For figure plates, Fireworks (Macromedia, Inc., San Francisco, CA), Volocity (Improvision, Inc., Lexington, MA), and Photoshop (Adobe Systems, San Jose, CA) were used to compose images from digital files in which all optical sections for each physical section were flattened into one plane. Images were enhanced only for contrast and brightness.

Control measures

Several control measures were taken to verify that each fluorescent tracer labeled only the intended nerve. First, the geniculate ganglion, which contains cells whose axons make up either the GSP or the CT nerve (never both; Fig. 1), were removed from three triple-labeled control and three sodium-restricted rats, sectioned on a cryostat, resected as described above, and examined with the confocal laser microscope for the presence of double labeling. Because the tracer was applied peripherally to the ganglia, these cells were labeled as well. Therefore, double-labeled geniculate ganglion cells would indicate a contamination of one nerve label with the other label (e.g., contamination of the GSP label with the CT label). In all cases, no double-labeled ganglion cells were observed. The labeling site of the IX nerve was distant from the tympanic bulla; therefore, petrosal ganglia were not examined.

Second, in addition to labeling afferent fibers that terminate in the NTS, each fluorescent tracer is effective in retrogradely labeling the respective salivatory nucleus cells (Contreras et al., 1980). These cells can be seen closely apposed medially to the intermediate and ventral zones of the NTS (Fig. 3B–D,G,H) and send outputs through only one nerve. We examined these retrogradely labeled cells for the presence of single or multiple fluorescent labels in every animal in which terminal field data were collected. Again, double-labeled cells would indicate that a single tracer could inadvertently label more than one nerve. Upon every observation of the labeled terminal fields, the retrogradely labeled salivatory neurons never contained a double label.

Finally, the caudal portion of the NTS was examined in all brains to determine whether the vagus nerve was inadvertently labeled (Hamilton and Norgren, 1984). In no case was there fluorescent label in the area of the NTS receiving vagal inputs.

NTS volume

To gain a perspective on the extent of terminal field distributions within the solitary nucleus, a myelin stain was used to determine total NTS volume (Fig. 3I–L). Separate groups of adult control ($n = 4$) and sodium-restricted ($n = 4$) rats aged 40–60 days were perfused, and brains were sectioned horizontally at 25 μ m on a vibratome as described above. Sections were mounted on slides, delipi-

dated in a graded series of alcohol and chloroform, and stained with a mixture of chromoxane cyanine R and 0.21 M ferric chloride (Kiernan, 1984). With the exception of the solitary tract, the NTS is a myelin-poor area whose surrounding structures are myelin rich. The darkly stained myelin allows the boundary of the NTS to be visualized throughout its dorsal-ventral extent (Fig. 3I–L). To quantify NTS volumes, the visible perimeter of the right NTS was outlined in each section, and the corresponding areas were calculated in the NeuroLucida software. Total volume of the right NTS was determined by summing the areas of each section that contained terminal field (~22) and multiplying by 25 (25 μm was the thickness of each section).

Ganglion cell quantification

To determine the effects of sodium restriction on the survival of primary taste neurons, separate groups of control ($n = 10$) and sodium-restricted ($n = 10$) rats were used to count individual cells in the geniculate and petrosal ganglia. The CT, GSP, and IX nerves were each labeled individually in separate rats as described above, except that Microruby (Molecular Probes) was used as the anterograde tracer. Microruby was used over other tracers because of its superior quality in labeling ganglion cells and because it can be used to visualize labeled cells by imaging intact (i.e., unsectioned) ganglia (Shuler et al., 2004). Visualization of BDA and Cascade blue require histochemical reactions for visualization with the confocal laser microscope (see above under Tissue preparation). Each rat was perfused as described above. Ganglia were removed and postfixed in 8% paraformaldehyde overnight. Each ganglion was then dissected from either the VII or the IX nerve, and serial optical sections of intact ganglia were imaged on the confocal microscope at 2- μm intervals with the green laser, generating an average of 50 optical sections through each ganglion. All labeled neurons were physically counted with the aid of NeuroLucida software.

Statistical analysis

Mean total terminal field volume for each nerve and terminal field overlap volumes among nerves were compared statistically between control and sodium-restricted adults by analysis of variance (ANOVA). Mean volumes of dorsal, intermediate, and ventral portions (see Results for definitions of these zones) were also calculated, compared between control and sodium-restricted rats, and analyzed via ANOVA. In addition, ANOVAs were used to analyze statistical differences in NTS volumes and total ganglion cell numbers between control and sodium-restricted rats. All values are expressed as mean \pm SE. Statistical results with an alpha level less than 0.05 were reported as significant.

RESULTS

Total terminal field volumes

Controls. Figure 4 shows the mean total volumes of terminal fields for the GSP, CT, and IX nerves. In control rats, the GSP terminations occupied the largest proportion of the NTS, with a mean (\pm SEM) total terminal field volume of $77.3 \pm 11.9 \times 10^6 \mu\text{m}^3$. Total mean CT terminal field volume was $31.0 \pm 3.0 \times 10^6 \mu\text{m}^3$, and IX terminals occupied a mean volume of $67.2 \pm 14.5 \times 10^6 \mu\text{m}^3$. In

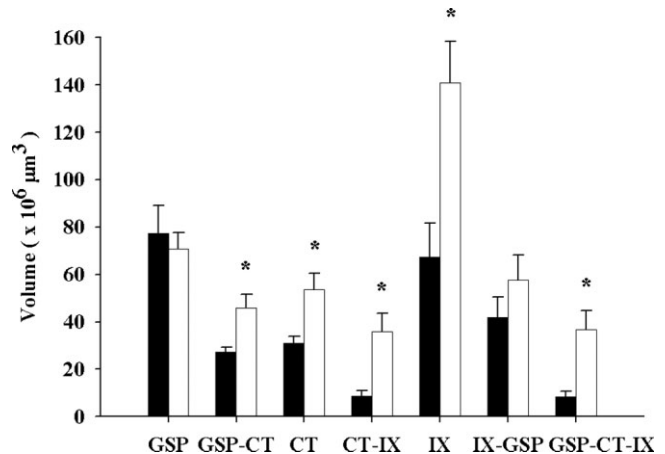


Fig. 4. Mean (\pm SEM) total terminal field volumes of the GSP, CT, and IX nerves and corresponding overlap (e.g., GSP-CT) in the NTS of control (solid bars) and sodium-restricted (open bars) rats. In restricted rats, the CT and IX fields are larger than those of controls. This expansion results in corresponding increases in the overlap among terminal fields. GSP field size does not change. * $P < 0.05$.

controls, GSP terminations began more dorsally than did CT terminals, and GSP terminal fields extended to (or beyond) the ventral extent of the CT terminal fields (Fig. 3A–D). IX projections terminated even more dorsally than GSP and extended ventrally to the dorsalmost portion of CT field (Fig. 3A,B), with the densest portions occupying the medial NTS. Therefore, the sequence of terminal fields in the control NTS in order of terminations from dorsal to ventral was IX, GSP, and then CT.

These three individual fields overlapped extensively with each other. CT and GSP fields overlapped widely within the rostral pole of the NTS (Fig. 3B–D), with a mean (\pm SEM) overlap volume of $27.2 \pm 2.0 \times 10^6 \mu\text{m}^3$ (Fig. 4). This represents approximately 88% of the CT field overlapping with the GSP field. A greater absolute amount of overlap occurred between GSP and IX fields ($41.8 \pm 8.6 \times 10^6 \mu\text{m}^3$) than for CT and IX fields ($8.6 \pm 2.4 \times 10^6 \mu\text{m}^3$; Fig. 4); however, the percentage of overlap that the GSP field had with the IX field and the percentage of overlap the CT field had with the IX field were not as extensive as was the percentage of overlap between the CT and the GSP fields (62% for GSP and IX overlap and 28% between CT and IX). The ventralmost extension of the IX field did not extend much beyond the dorsalmost boundary of the CT field, whereas the dorsalmost boundary of the GSP field invaded the ventral portion of the IX terminal field (Fig. 3A,B). The total mean volume for the overlap among all three fields was $8.5 \pm 2.3 \times 10^6 \mu\text{m}^3$ (Fig. 4). This overlap was essentially the amount found for the CT field overlap with the GSP (Fig. 4).

Sodium-restricted rats. Developmental dietary sodium restriction resulted in profound alterations in the topography and distribution of gustatory terminal fields. Early dietary sodium restriction resulted in a significantly enlarged mean (\pm SEM) CT field ($53.6 \pm 6.7 \times 10^6 \mu\text{m}^3$) compared with controls ($P = 0.005$; Fig. 4). This nearly twofold enlargement occurred in the dorsalmost portion of the CT terminal field, as it expanded caudally into the territories of the GSP and IX fields (Figs. 2E, 3F). The

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GSP terminal field in sodium-restricted rats was similar to controls ($70.7 \pm 7.0 \times 10^6 \mu\text{m}^3$; $P = 0.65$; Fig. 4). Finally, the total IX field volume was over two times greater than controls ($140.9 \pm 17.5 \times 10^6 \mu\text{m}^3$; $P = 0.013$; Fig. 4). The IX field extended dorsally, ventrally, caudally, and laterally, well past the control IX field (Fig. 3E–H).

The amount of overlap occurring among fields was also different from that in controls. As the CT and IX terminal fields increased in size, they expanded beyond the areas normally occupied in controls. Indeed, the volume of the mean (\pm SEM) CT-GSP field overlap was significantly greater than that in controls ($46.0 \pm 5.6 \times 10^6 \mu\text{m}^3$; $P = 0.004$; Fig. 4). Furthermore, the increase in overlapping volume was proportionate to the increase in volume of the CT field in sodium-restricted animals and did not differ between groups (88% vs. 86% of the CT field overlapped with the GSP field in controls and restricted rats, respectively). The overlap between the CT and IX fields also significantly increased with the dietary manipulation ($35.8 \pm 7.7 \times 10^6 \mu\text{m}^3$; $P = 0.007$; Fig. 4) and was reflected in a larger proportion of the CT field overlapping with the IX field (28% vs. 67%). However, there were no group-related differences in the IX-GSP field overlap ($57.6 \pm 10.7 \times 10^6 \mu\text{m}^3$; $P = 0.280$; Fig. 4), although the percentage of GSP field contained within the IX field was slightly greater in restricted rats (62% vs. 82%). This indicates that the CT and IX fields extended past territory occupied in controls, into neighboring terminal fields. Finally, there was a significant increase in the volume in which all three terminal fields were common ($36.7 \pm 8.1 \times 10^6 \mu\text{m}^3$; $P = 0.007$; Fig. 4), again primarily because of the increased size of the CT field.

Zonal distribution of terminal fields

To localize regions especially susceptible to the dietary manipulation and to be consistent with our previous studies of CT terminal field in the NTS (King and Hill, 1991; Krimm and Hill, 1997; Sollars et al., 2006), the NTS was divided into dorsal, intermediate, and ventral zones. The dorsal zone contained sections in which the solitary tract was most visible and included sections in which the fourth ventricle occupied the largest medial-lateral extent (Fig. 3I,J). The dorsal zone was further characterized by the spinal trigeminal tract extending to approximately the rostralmost extent of the NTS and by the lack of the hypoglossal nucleus and the facial nucleus (Fig. 3I,J; facial nucleus not shown). This was the largest of the three zones, encompassing almost the entire IX terminal field in controls (Fig. 3A,B). In controls, this zone typically consisted of four or five 50- μm sections and was distinguished by the round CT terminal field (Figs. 2B, 3B). The intermediate zone (usually two 50- μm sections) was characterized by the decrease in the fourth ventricle volume compared with the dorsal zone, by the dorsal extent of the hypoglossal nucleus, by the extension of the spinal trigeminal tract rostrally beyond the inferior cerebellar peduncle, and by the presence of the dorsal extent of the facial nucleus (Fig. 3K; facial nucleus not shown). In all animals, regardless of group, the terminal fields of the CT and GSP in the intermediate zone was most easily identified and most consistent across animals (Fig. 3C,G). The ventral zone of the gustatory terminal fields (usually three or four 50- μm sections) had an expanded hypoglossal nucleus and facial nucleus compared with the intermediate sections (Fig. 3L). Dividing the fields in this manner highlights the

vastly different distribution of terminations along the dorsal-ventral aspect of the NTS and more narrowly defines specific regions of anatomical change. Compartmentalizing terminal fields into zones also demonstrates the similar characteristics and contiguous and overlapping arrangement of the three afferent terminal zones.

It must be pointed out that we use the terms “dorsal,” “intermediate,” and “ventral” zones here to be consistent with earlier reports (King and Hill, 1991; Krimm and Hill, 1997; Sollars et al., 2006). However, the orientation of the NTS within the brainstem is such that the caudal portion of the NTS is dorsal to the ventralmost portion. That is, the NTS is inclined from rostral to caudal. Therefore, the “dorsal” zone more accurately represents the dorsal-caudal portion of the field in the NTS and the intermediate, and ventral sections represent a more ventral-rostral portion of the terminal field in the NTS.

Dorsal zone terminal field volume. The dorsal zone of the NTS exhibited the most substantial plasticity compared with other regions of the NTS. Consistently with previous findings (King and Hill, 1991; Krimm and Hill, 1997; Sollars et al., 2006), the mean (\pm SEM) volume of the CT field was over twice as great in the sodium-restricted animals ($32.0 \pm 5.9 \times 10^6 \mu\text{m}^3$) compared with controls ($15.6 \pm 2.0 \times 10^6 \mu\text{m}^3$; $P = 0.023$; Fig. 5A). The labeled fibers in restricted rats appeared more densely packed and extended more caudally than in controls (Figs. 2B,E, 3A,B,E,F). The corresponding portion of the GSP field was not significantly different between groups (restricted: $57.4 \pm 10.7 \times 10^6 \mu\text{m}^3$ vs. control: $72.0 \pm 13.5 \times 10^6 \mu\text{m}^3$; $P = 0.442$; Figs. 2A,D, 5A). However, the volume of the IX field underwent a significant expansion in sodium-restricted rats compared with controls ($131.8 \pm 20.0 \times 10^6 \mu\text{m}^3$ vs. $67.0 \pm 14.3 \times 10^6 \mu\text{m}^3$; $P = 0.030$; Fig. 5A). The expansion of the IX field in the dorsal region extended farther laterally, caudally, and ventrally than in control animals (Figs. 2C,F, 3A,B,E,F).

Such differences in the patterns of gustatory terminations in the dorsal NTS were also reflected in the proportions of the overlapping fields. The dorsal CT field overlapped completely within the GSP field, and this volume of overlap was significantly greater in restricted rats compared with controls ($28.3 \pm 5.6 \times 10^6 \mu\text{m}^3$ vs. $14.9 \pm 2.0 \times 10^6 \mu\text{m}^3$; $P = 0.041$; Figs. 3B,F, 5A). In fact, the increase in overlap between the two fields was directly related to the increase in volume of the CT field, in that the overlap increased proportionally to the increase in CT terminal field size. There was also a significant increase in the overlap between the CT and the IX fields when comparing restricted with control rats ($28.7 \pm 5.2 \times 10^6 \mu\text{m}^3$ vs. $8.6 \pm 2.4 \times 10^6 \mu\text{m}^3$; $P = 0.007$; Figs. 3B,F, 5A). This increase also was due to the expansion of both the CT and the IX fields past their normal boundaries. However, there was no significant difference in the volume of the overlapping regions of the GSP and IX fields (restricted: $49.9 \pm 9.2 \times 10^6 \mu\text{m}^3$ vs. control: $41.7 \pm 8.5 \times 10^6 \mu\text{m}^3$; $P = 0.533$; Figs. 3B,F, 5A). Finally, the volume of the region in which of all three terminal fields overlapped also increased significantly (restricted: $28.2 \pm 5.6 \times 10^6 \mu\text{m}^3$ vs. control: $8.4 \pm 2.3 \times 10^6 \mu\text{m}^3$; $P = 0.009$; Figs. 3B,F, 5A).

Intermediate zone terminal field volume. A different pattern of terminal field organization between groups was also evident in the intermediate zone of the NTS. Here, the CT terminal fields in both groups had a more compact oval configuration than in dorsal regions (Fig.

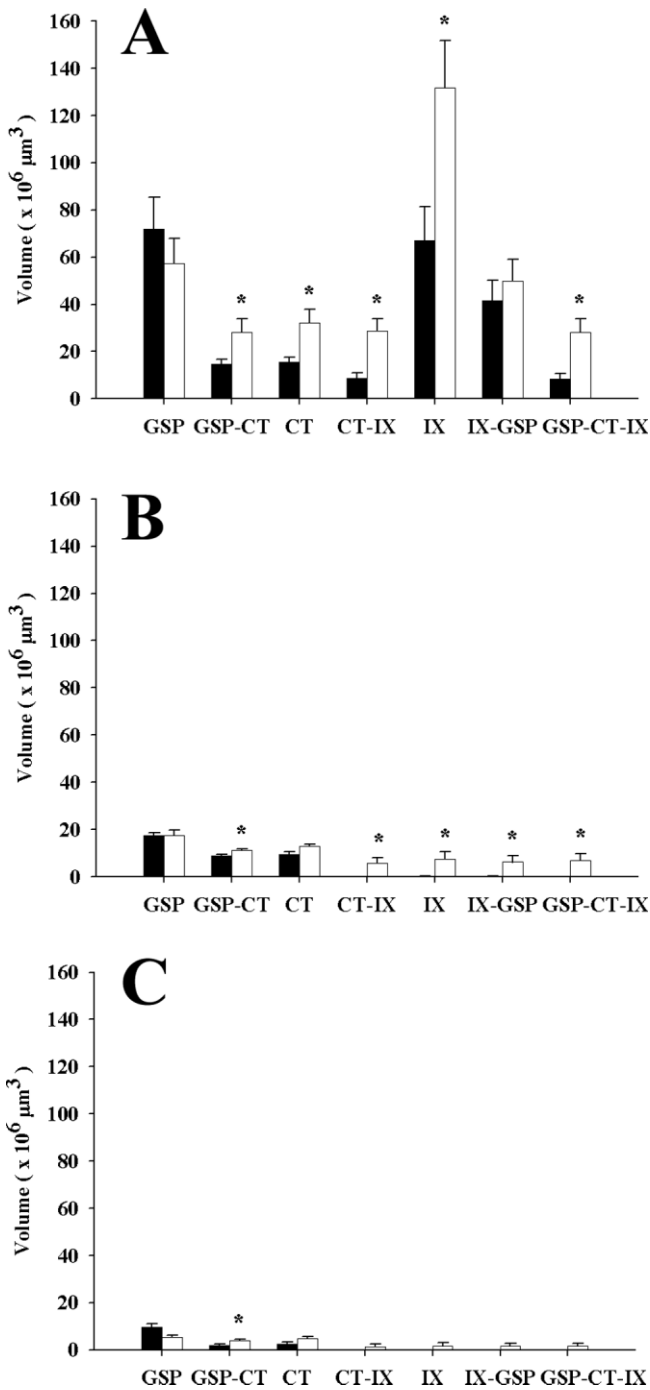


Fig. 5. Zonal distribution of mean (\pm SEM) terminal field volumes of the GSP, CT, and IX nerves and corresponding overlap in the NTS of control (solid bars) and restricted (open bars) rats. **A:** Dorsal zone. The largest proportion of the terminal fields is contained in the dorsal zone. Also, the greatest increases in terminal field volume occur in this zone in restricted rats compared with controls. **B:** Intermediate zone. Terminal field volumes are distributed differently in this zone compared with others. Dietary manipulation affects only the IX field and resulting overlap of the IX field with CT and GSP. **C:** Ventral zone. This zone contains the lowest volume of terminal fields. The significance in CT-GSP overlap in restricted rats is attributed to a difference in how the GSP and CT fields are distributed within the NTS. * $P < 0.05$.

3C,G). In the intermediate zone, the volume of CT terminal field label in restricted animals was not significantly different from control ($12.7 \pm 1.0 \times 10^6 \mu\text{m}^3$ vs. $9.4 \pm 1.1 \times 10^6 \mu\text{m}^3$; $P = 0.069$; Fig. 5B). Furthermore, the GSP field volumes in this zone were also similar between groups (restricted: $17.4 \pm 2.4 \times 10^6 \mu\text{m}^3$ vs. control: $17.5 \pm 1.1 \times 10^6 \mu\text{m}^3$; $P = 0.978$; Fig. 5B). However, the IX field in sodium-restricted rats showed robust expansion into the intermediate portion of the NTS, extending ventrally beyond the boundaries of the control field; in controls, the IX field was not present in the intermediate NTS (Fig. 3C,G). Specifically, sodium restriction yielded a 40-fold increase in volume compared with controls ($7.4 \pm 3.0 \times 10^6 \mu\text{m}^3$ vs. $0.2 \pm 0.2 \times 10^6 \mu\text{m}^3$; $P = 0.030$; Fig. 5B).

Despite no significant changes in the total volume of CT and GSP terminal fields in the intermediate NTS, there was a significant increase in the volume of overlap between the GSP and the CT fields in restricted rats compared with controls ($11.1 \pm 0.6 \times 10^6 \mu\text{m}^3$ vs. $8.7 \pm 0.7 \times 10^6 \mu\text{m}^3$; $P = 0.039$; Figs. 3C,G, 5B). Additionally, the volume of the overlapping field both between CT and IX fields and between GSP and IX fields in restricted rats increased proportionally with an increase in the IX terminal field volume (CT-IX restricted: $5.6 \pm 2.5 \times 10^6 \mu\text{m}^3$ vs. control: $0.004 \pm 0.004 \times 10^6 \mu\text{m}^3$; $P = 0.04$; IX-GSP restricted: $6.1 \pm 2.7 \times 10^6 \mu\text{m}^3$ vs. control: $0.1 \pm 0.1 \times 10^6 \mu\text{m}^3$; $P = 0.04$; Fig. 5B). Thus, in restricted rats, the IX terminal field in the intermediate zone of the NTS intersected with both the GSP and the CT terminal fields. This is also evident in the significantly increased volume of the overlapping portion of all three terminal fields (restricted: $7.0 \pm 2.8 \times 10^6 \mu\text{m}^3$ vs. control: $0.004 \pm 0.004 \times 10^6 \mu\text{m}^3$; $P = 0.03$; Figs. 3C,G, 5B).

Ventral zone terminal field volume. The ventral portion of the NTS contained the least volume of gustatory terminal fields. Both CT and GSP terminal fields were sparse and were not reliably different between diet conditions ($P = 0.069$ and $P = 0.052$, respectively; Fig. 5C). In two of the nine sodium-restricted rats, a small portion of the IX field extended into the ventral NTS; this extension occurred in none of the control rats (Fig. 3D,H). However, there was not a significant difference in the volume of the IX field in sodium-restricted rats compared with controls ($P = 0.152$; Fig. 5C).

In addition, the terminal field volume overlap between CT-IX and IX-GSP were not significantly different between groups ($P = 0.185$ and $P = 0.184$, respectively; Fig. 5C). However, there was a significant increase in the overlap of the GSP and CT terminal fields in sodium-restricted rats compared with controls ($P = 0.042$; Figs. 3D,H, 5C). Finally, the overlap among all three terminal fields was not significantly different ($P = 0.189$; Fig. 5C).

Coronal sections: an illustration of the overlapping fields in subnuclei. As noted previously, the NTS is inclined dorsoventrally as it extends rostrally; therefore, the term "dorsal" also denotes caudal. This feature is best seen through serial coronal sections of the brainstem. Moreover, subdivisions of the NTS based on cyto- and chemoarchitectural features have been defined exclusively with coronal sections (Whitehead, 1988; Halsell et al., 1996; Zhang and Ashwell, 2001).

To illustrate the overlapping fields and the diet-related differences further, we labeled the gustatory nerves of a control and a developmentally sodium-restricted rat as was done earlier, but sections were made coronally

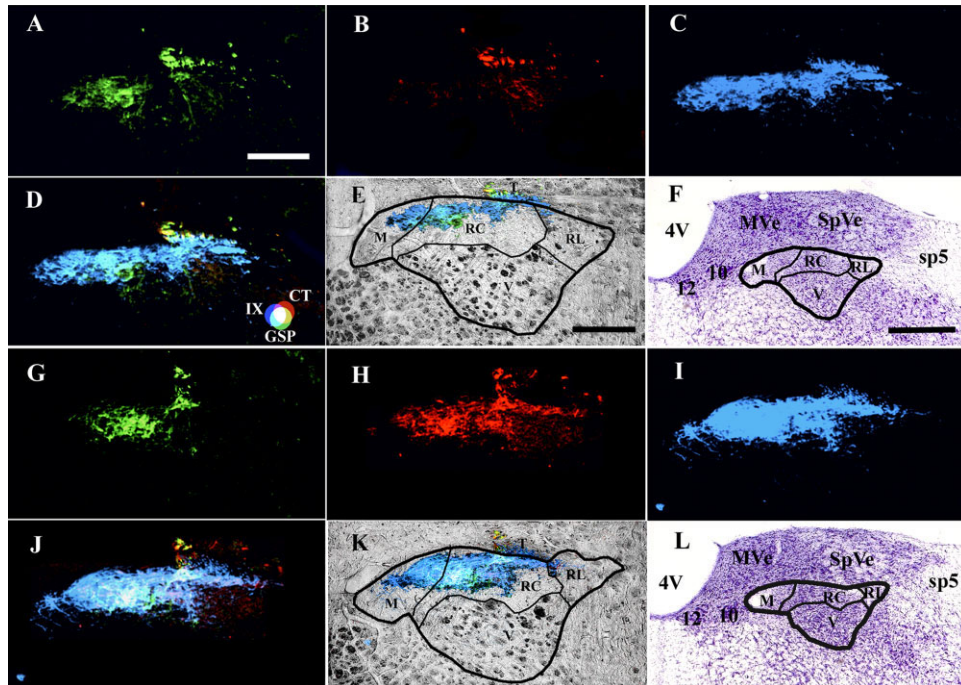


Fig. 6. Coronal section through the “intermediate” zone of the rat medulla in a control (A–F) and in a sodium-restricted rat (G–L) showing the terminal field for the GSP (A,G), CT (B,H), and IX (C,I) nerves. D and J show the merged terminal fields, and E and K show the merged image superimposed on an image of the brainstem obtained through the transmitted light channel on the confocal laser microscope system. F and L show Nissl-stained tissue illustrating the location of the NTS relative to other brainstem structures. Lines in E, F, K, and L demarcate the approximate boundaries of the subdivisions in the NTS as described by Halsell et al. (1996). These examples illustrate the primary findings from the data obtained in horizontal sections: GSP terminal fields are similar between control (A) and sodium-restricted (G) rats, whereas the CT terminal field in sodium-restricted rats (H) extends more dorsally and caudally than in controls (B), and the IX terminal field is enlarged and extends more ventrally in sodium-restricted rats (I) than in controls (C). The ex-

panded CT and IX fields in sodium-restricted rats do not invade additional subdivisions in the NTS compared with control (E,K). Note that the borders of the subdivisions are close approximations of what the borders are as revealed by more extensive staining procedures (see Halsell et al., 1996). F and L show that these sections are from the “intermediate” zone and are from approximately the same rostral-caudal level. Refer to the color key in D to identify individual fields and overlap among different terminal fields. D, dorsal; L, lateral; T, solitary tract; M, medial subdivision of the NTS; RC, rostral central subdivision of the NTS; V, ventral subdivision of the NTS; RL, rostral lateral subdivision of the NTS; 4V, fourth ventricle; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; MVe, medial vestibular nucleus; sp5, spinal trigeminal tract; SpVe, spinal vestibular nucleus. Scale bars = 200 μ m in A (applies to A–D,G–J); 200 μ m in E (applies to E,K); 500 μ m in F (applies to F,L).

through the NTS. Terminal field labeling and imaging of the GSP, CT, and IX were accomplished as described for the horizontally sectioned tissue (Fig. 6A–D,G–J). Additionally, one optical section using transmitted light was imaged to determine subnuclei within the NTS. This allowed us to overlay accurately the flattened terminal field image onto an image in which subnuclei of the NTS could be visualized (Fig. 6E,K). Finally, each section was mounted on slides and subsequently stained with creyl violet to show the NTS and other dorsal medullary structures (Fig. 6F,L). No measurements were taken from coronal sections.

Figure 6 shows the overlapping fields in a control (Fig. 6A–F) and in a developmentally sodium-restricted (Fig. 6G–L) rat. These coronal sections were taken approximately 600 μ m caudal from the anteriormost extent of the NTS (Fig. 6F,L). Therefore, sections used here to illustrate the diet-related effects were from the “intermediate” zone of the NTS (Halsell et al., 1996). The GSP terminal fields were similar between the control and the sodium-restricted rats (Fig. 6A,G). However, the CT terminal field in the sodium-restricted rat (Fig. 6H) was substantially

larger than that in the control rat (Fig. 6B). Similarly, the IX terminal field in the sodium-restricted rat (Fig. 6I) was larger and extended more ventrally than that in the control rat (Fig. 6C). These fields combined to show greater amounts of overlap in the sodium-restricted rat (Fig. 6J) compared with the control rat (Fig. 6D). The expanded terminal fields in the sodium-restricted rat were contained within the same subnuclei as in the control. That is, the labels were limited primarily to the rostral-central and medial subnuclei and did not extend into the ventral subnucleus (Fig. 6E,K). It should be noted that the boundaries for the subdivisions of NTS subnuclei shown in Figure 6 are similar to those shown by Halsell et al. (1996); the exact boundaries may differ slightly from the boundary shown.

Summary of terminal field organization. The three gustatory terminal fields in control rats were arranged in an ordered, yet overlapping, fashion such that IX terminals began most dorsally and spread rostrally, caudally, laterally, and ventrally into the GSP terminal field. As the IX field reached its ventralmost extent, the CT field began and overlapped almost completely with the GSP terminal

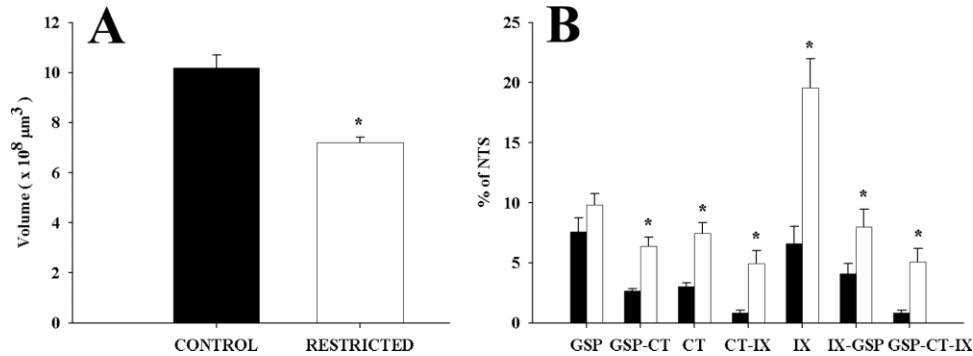


Fig. 7. **A:** Total mean volume of the right half of the NTS in control (solid bars) and restricted (open bars) rats. The NTS volume of restricted rats is significantly lower than that of controls. **B:** Mean volume of GSP, CT, and IX terminal fields and their overlapping

portions normalized to total NTS volume in control (solid bars) and restricted (open bars) rats. A greater proportion of the NTS is occupied by overlapping terminal fields in restricted rats compared with controls. Error bars denote SEM. * $P < 0.05$.

field. The GSP field began more dorsally than the CT field, but both GSP and CT fields extended to the same ventral boundary of the NTS. Sodium restriction dramatically altered the topography of the CT and IX terminal fields of the NTS, without affecting the distribution of the GSP terminal field. For the CT terminal field, this plasticity was most evident in the dorsal portion of the CT field. The effects on the IX terminal field were more widespread; IX fields extended farther dorsally, ventrally, caudally, and laterally throughout the NTS. The three terminal fields overlapped more extensively in sodium-restricted rats compared with control rats, indicating that all three gustatory nerves converge onto larger areas in the NTS of restricted rats than in controls.

Total NTS volume

Because sodium-restricted rats are much smaller than controls at adulthood (Hill et al., 1986), we measured the total NTS volume in sodium-restricted and control rats to examine the relative size of the terminal fields within the NTS. The mean total volume of the right NTS was $10.2 \pm 0.5 \times 10^8 \mu\text{m}^3$ in control and $7.2 \pm 0.2 \times 10^8 \mu\text{m}^3$ in restricted rats. This represented a significant 29.2% decrease in total volume of NTS in sodium-restricted rats compared with controls ($P = 0.001$; Fig. 7A). These totals were consistent with the total NTS volumes reported by King and Hill (1991). When individual nerve terminal field volumes were standardized to their respective NTS volumes, the group-related differences were magnified. The CT and IX terminal fields and their respective overlapping portions occupied a significantly larger percentage of the nucleus relative to controls, whereas the GSP terminal field did not differ significantly between groups. The CT terminal field occupied 7.4% of the total NTS in sodium-restricted rats compared with 3% of the NTS in control rats ($P = 0.0001$; Fig. 7B). The IX terminal field occupied 19.5% of the total NTS in sodium-restricted rats compared with 6.6% of the NTS in control rats ($P = 0.002$; Fig. 7B). The GSP terminal field occupied 9.8% of the total NTS in sodium-restricted rats compared with 7.6% of the NTS in control rats ($P = 0.17$; Fig. 7B). The corresponding overlap between the individual fields and among all three fields also increased significantly (GSP-CT: $P = 0.0001$; CT-IX: $P = 0.004$; IX-GSP: $P = 0.047$; Fig. 7B). Therefore, the differences in the larger absolute volumes for the CT

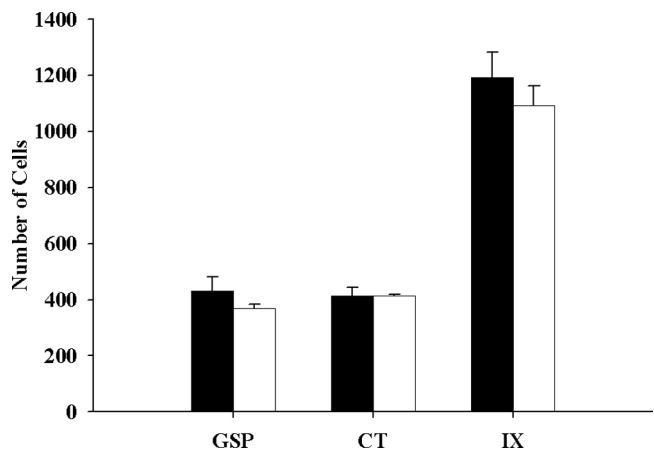


Fig. 8. Mean (\pm SEM) number of geniculate (GSP, CT) and petrosal (IX) ganglion cells in control (solid bars) and restricted (open bars) rats. The numbers of cells in each ganglion are similar in control and restricted rats. Note: petrosal ganglia contain three times more cells than geniculate ganglia.

and IX fields in sodium-restricted rats compared with controls were exaggerated when expressed relative to the smaller NTS.

Ganglion cell number

Ganglion cell numbers were counted to address the hypothesis that the increase in terminal field volumes in sodium-restricted rats was due to an increased number of primary afferents terminating in the NTS. That is, a diet-related increase in neuron number may result in an increase in the volume of afferent projections to the NTS. However, sodium restriction did not alter the total number of ganglion cells for any of the three taste nerves. Figure 8 shows that there were no significant differences between control and sodium-restricted ganglion cell numbers for the CT ($P = 0.105$), GSP ($P = 0.065$), or IX ($P = 0.931$) nerves.

DISCUSSION

Through the use of a novel triple-fluorescence labeling procedure, this study demonstrates specific and dramatic

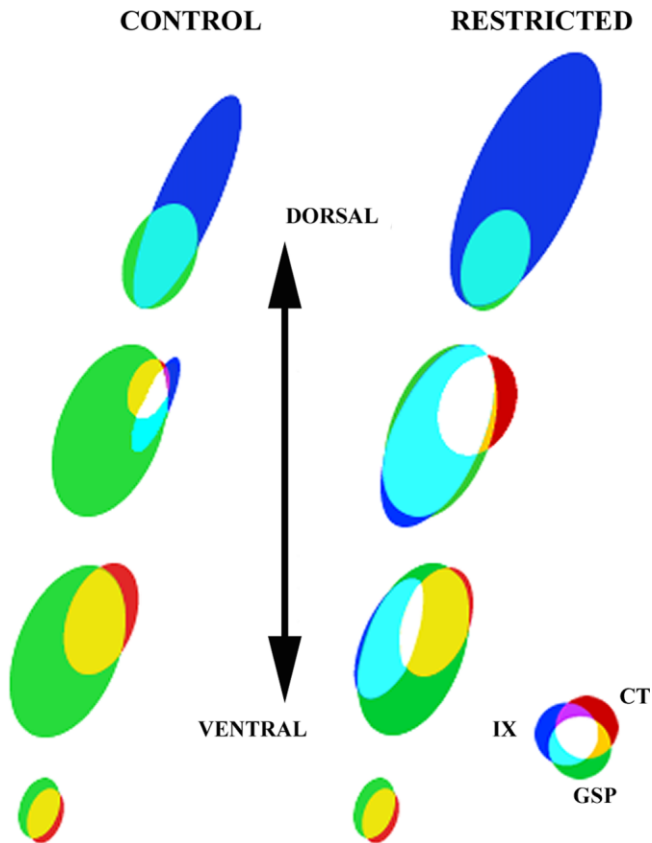


Fig. 9. Model of terminal field organization through the dorsal-ventral extent of horizontal sections from the right NTS in control and restricted rats. Overlapping fields are represented at four levels along the dorsal-ventral axis. See Results for details of the overlap among the three fields and a comparison of diet-related differences. Refer to color key to identify individual fields and overlap among different terminal fields. Note: Because of the orientation of the NTS within the brainstem, the term “dorsal” sections refers to dorsal-caudal, and “ventral” refers to ventral-rostral.

plastic anatomical changes in afferent terminal fields in the gustatory medulla. The total volumes of the CT and IX nerve terminal fields were expanded to at least twice their normal size in developmentally sodium-restricted rats, whereas the GSP was unaffected by the dietary manipulation. Moreover, the change in terminal field volumes affected the way in which nerves project into the NTS and overlap with other fields. These effects were even more dramatic when considering that the total volume of the NTS in restricted rats was less than in controls. Furthermore, this plasticity could not be accounted for by sheer increases in innervating afferent fibers, because sodium restriction did not alter the survival of geniculate or petrosal ganglion cells.

A clear advantage of our triple-labeling approach is that, for the first time, the relative relationships among the terminal fields of three distinct gustatory nerves could be examined in the medulla of controls and experimental animals. Figure 9 depicts this organization in both control and sodium-restricted rats. In control rats, the IX terminates dorsally to the other two terminal fields and projects ventrally to overlap with the dorsal (i.e., dorsal-caudal)

portion of the GSP field. The IX field extends more rostrally at the dorsal GSP zone and terminates more laterally in the NTS than was previously reported (Hamilton and Norgren, 1984; see also Fig. 3A,B). The ventral portion of the IX field abuts the dorsalmost portion of the CT field. CT and GSP fields nearly overlap completely within the rostral pole of the NTS. Contrary to previous results, not only do the coextensive CT and GSP fields within the rostral NTS occupy the lateral portions of the NTS but both extend medially as well (Hamilton and Norgren, 1984; see Fig. 3A–C,E,F). Notably, there is a relatively small region where all three nerves terminate.

In sodium-restricted rats, the dorsal portion of the CT terminal field expands caudally outside its normal boundaries into GSP and IX field territory. The IX field extends dorsally, caudally, laterally, and ventrally, well past the apparent boundary of control IX field. Consequently, the overlap occurring among adjacent fields also increases.

Comparisons with previous studies

Consistently with the findings of King and Hill (1991) and Krimm and Hill (1997), we found anatomical changes induced by early dietary sodium restriction in the dorsal portion of the CT terminal field. It is within this zone where changes in terminal fields are also affected developmentally by limited taste experience (Lasiter and Diaz, 1992; Lasiter, 1995), taste receptor cell damage (Lasiter and Kachele, 1990), and high-salt diets (Pittman and Contreras, 2002). Thus, a variety of experimental procedures show this zone to be especially susceptible to environmental alterations.

However, our triple-label experiments show that plasticity is not exclusive to the CT terminal field as previously demonstrated (King and Hill, 1991) but also includes the IX terminal field. Furthermore, in contrast to previous reports that dietary sodium restriction does not change the absolute volume of the CT terminal field but changes the distribution of the terminal field in the NTS (King and Hill, 1991), we found that the dietary manipulation also increases the absolute CT terminal field volume. We believe that these differences are due partially to the relatively high sensitivity of labeling terminal fields with fluorescent tracers and to viewing fields with a confocal laser microscope.

Speculations on plasticity mechanisms

Because sodium-taste-elicited activity is a major component of the electrophysiological response of the CT nerve, hypotheses that center around CT activity shaping terminal field development have been attractive. Indeed, dietary NaCl restriction during early development produces a selective reduction in the taste response of the CT nerve to sodium salts but not to other taste stimuli (Hill, 1987; Hill and Przekop, 1988). Our data indicate, however, that terminal field plasticity is no longer unique to the CT field. In fact, compared with the CT, sodium restriction produces much more dramatic changes in the terminal field of the IX. However, the sensitivity of the IX nerve taste responses to sodium salts is insubstantial compared with responses of the CT nerve (Formaker and Hill, 1991; Kitada et al., 1998). Furthermore, the processes that determine the abnormal terminal fields in the NTS induced by dietary sodium restriction appear to be accomplished before taste receptor cells are present on the tongue. Restricting maternal dietary sodium during only a

brief, critical period from E3 to E12 is sufficient to cause an enlarged CT terminal field (Krimm and Hill, 1997). Thus, the parameters allowing plasticity to occur are established well before sodium taste stimuli are transduced through functional taste receptor cells, suggesting that an activity-dependent mechanism might not be the only explanation for alterations in terminal field development as a result of sodium restriction. Dietary sodium restriction must influence gustatory nerve terminal field development by additional, nonactivity-dependent mechanisms as well.

Prenatal dietary manipulations have a range of physiological consequences that extend beyond the gustatory system and may play a role in gustatory terminal field development. For example, reduction of sodium intake affects hormonal levels such as aldosterone (Kifor et al., 1991; Lehoux et al., 1994) and early-dietary-restriction paradigms influence levels of growth factor such as IGF1 (Fliesen et al., 1989). Indeed, IGF1 has significant effects on NTS development (Eustache et al., 1994; Dentremont et al., 1999). Thus, the anatomical rearrangement occurring within the NTS might not be directly related to taste function but might result from nonspecific effects of early sodium restriction. That is, the boundaries and subsequent molecules associated with boundary formation for the CT and IX field (e.g., axonal pathfinding) may be altered through early dietary sodium restriction, independently of neural activity.

Some selectivity must still be present, however, because the GSP terminal field is not affected. It is possible that the effects of early dietary manipulation may be expressed after processes that determine GSP field development are solidified. That is, the processes resulting from our dietary manipulation may exist after the GSP field is determined but before the molecular cues associated with the formation of CT and IX terminal fields are organized.

Given the development of the three terminal fields, it is possible that the GSP field may shape the formation of other gustatory fields in the NTS, especially the CT. Facial nerve axons, which include both CT and GSP, terminate in the rostral NTS at P1 and develop to approximately P25 (Lasiter, 1992). IX fibers enter the NTS around P10 and develop to approximately P45 (Lasiter, 1992). This chronological pattern of innervation and terminal field formation in the NTS occurs in conjunction with the morphological development of gustatory receptors. The maturation of palatal taste receptor cells precedes that of fungiform and circumvallate taste receptor cells (Harada et al., 2000). Furthermore, preliminary data from our laboratory indicate a significant GSP field development by P10, whereas CT field development is immature at this age (S.I. Sollars and D.L. Hill, unpublished observations). Given this sequence of individual terminal field development, we propose that the GSP terminations arrive first in the NTS and establish their boundaries before the effects of the plasticity-inducing diet are realized. As the GSP terminal field becomes established, the CT terminal field termination pattern is subject to the milieu organized by the pioneering GSP field. Indeed, the restricted CT terminal field pattern mirrors that of the GSP nerve; they overlap almost entirely within the rostral pole of the NTS. As an aside, this would predict that disruption in GSP field development would correspondingly disrupt CT field development. Finally, the IX fibers, perhaps as a result of

influences from other nerves, expand to where the boundary cues are apparently grossly disrupted.

Potential functional and behavioral effects

As a result of the disruption of individual terminal field boundaries, gustatory afferents from the three nerves likely converge anatomically more onto NTS neurons than in controls. Such convergence might have a corresponding functional basis. In addition to the selective decrease in taste responses from NTS neurons driven by stimulation of the anterior tongue in sodium-restricted rats (i.e., CT stimulation; Vogt and Hill, 1993), functional alterations might also be evident in NTS neurons receiving unique combinations of two or three afferent inputs. Our data indicate that NTS neurons in sodium-restricted rats would likely receive inputs from receptive fields not normally found in controls (Travers et al., 1986). As a corollary to the unusual synaptic inputs in restricted rats, the morphology of NTS neurons and associated synaptic structures would also likely be affected. Indeed, gross changes in dendritic length and number occur in sodium-restricted rats (King and Hill, 1993). This potential anatomical and functional convergence might also be reflected behaviorally by altering taste-related behaviors, including sodium appetite. Therefore, the striking alterations occurring in the NTS both pre- and postsynaptically as a result of developmental dietary manipulation have important implications for central processing of taste stimuli and for behavior.

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