Forebrain neurons that project to the gustatory parabrachial nucleus in rat lack glutamic acid decarboxylase

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MATERIALS AND METHODS

Subjects. Five male Sprague-Dawley (SD) rats weighing 350–450 g [CrL:CD (SD) BR; Charles River Breeding Laboratories] were used in this study. The animals were maintained in a temperature-controlled colony room on a 12:12-h light-dark cycle and allowed free access to normal rat chow (Teklad 8604) and distilled water. All procedures conformed to National Institutes of Health guidelines and were approved by the University of Louisville Institutional Animal Care and Use Committee.

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Surgery. The rats were anesthetized with a 50 mg/kg ip injection of pentobarbital sodium (Nembutal). Atropine was administered to reduce bronchial secretions. Additional doses of Nembutal (0.1 ml) were administered as necessary to continue a deep level of anesthesia. The animals were placed on a feedback-controlled heating pad, and rectal temperature was monitored to maintain body temperature at 37 ± 0.5°C. Animals were secured in a stereotaxic instrument, and the skull was exposed with a midline incision then leveled with reference to β and λ. A small hole was drilled through the bone overlying the cerebellum to allow access to the PBN.

Electrophysiological recording. A 0.1 M NaCl solution was applied to the anterior two-thirds of the tongue for 10 s using a wash bottle, and extracellular neural responses were recorded using a glass-insulated tungsten microelectrode oriented 20° off vertical with the tip pointing rostral (1–3 MΩ). Only the anterior two-thirds of the tongue was stimulated because numerous prior studies have demonstrated that forebrain activation has a profound influence on brain stem taste cells that receive input via the chorda tympani nerve (8, 21–24, 37, 38). Further, the concentration of NaCl used in the present study has been shown to produce a significant neural response in each “best-stimulus” class of PBN neurons (23, 24). Once the gustatory PBN was located, the tungsten electrode was replaced by a micropipette (ID 10–20 μm) filled with 4% Fluorogold (FG; Biotium) dissolved in saline. The taste-responsive area was electrophysiologically relocated, and FG was iontophoretically injected (+2 μA for 20 min; 2 min on and 1 min off). Cambridge Electronic Design’s Spike2 hardware and software were used to record NaCl-evoked neural responses (23). No attempt was made to isolate single neurons for analysis of response rate; rather, we isolated regions in which a response to NaCl applied to the tongue was visually above the baseline discharge (Fig. 1A).

Colchicine treatment. Five days after surgery, the animals were reanesthetized and 3 μl of colchicine (20 μg/μl dissolved in 0.1 M NaCl, Tokyo Kasei) was infused into the lateral ventricle ipsilateral to the FG injection site [Coordinates: 0.85 mm anterior to bregma, 1.5 mm lateral to midline and 4.0 mm ventral to dura according to Paxinos and Watson (32)] using a 25-μl Hamilton syringe with a 28-gauge needle mounted in a stereotaxic microinjection unit (Kopf; model no. 5000). The animals were perfused 48 h after colchicine administration.

Perfusion and histology. The animals were administered a lethal dose of Nembutal (100 mg/kg ip) and perfused through the ascending aorta, initially with 250 ml of 0.9% saline containing 5 ml of 100 units/ml heparin followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, blocked just rostral to the PBN, and postfixied overnight at 4°C in the same fixative. Coronal (50 μm) sections were cut using a vibrating microtome (Leica VT 1000S), and every other section was collected for subsequent immunohistochemistry.

Immunohistochemistry. First, brain sections were incubated in 5% normal goat serum (NGS; Jackson Laboratories) and 1% BSA (Jackson Laboratories) mixed in 0.3% Triton-X phosphate-buffered saline (TPBS) for 1 h. Sections were then incubated overnight (4°C) in rabbit FG antibody (Chemicon; AB153) diluted 1:2,500 in TPBS. Sections were rinsed several times in PBS followed by 2-h incubation in FITC goat anti-rabbit diluted 1:100 in PBS containing 5% normal mouse serum (NMS; Jackson Labs) and 1% BSA for 1 h, then for 48 h at 4°C in mouse monoclonal glutamic acid decarboxylase antibody (Chemicon, catalog no. MAB5406) diluted 1:2,000 in PBS (36, 40). The immunogen for this antibody was a recombinant fusion protein containing the unique N terminal regions of GAD67 not shared by GAD65 protein. On day 4, sections were washed several times and incubated for 2 h in Cy-3 goat anti-mouse (Jackson Laboratories) diluted 1:200 in PBS containing 5% NMS. Following several rinses, the tissue sections were mounted on gelatin-coated slides and covered with a cover slip. For one series of tissue sections, the GAD-67 antibody was omitted and, consequently, Cy-3 immunofluorescence.

Data analysis. Cell bodies positive for FG (FITC; excitation filter: 490 nm; barrier filter: 550 nm) and GAD-67 (Cy-3; excitation filter: 520–554 nm; barrier filter: 580 nm) immunoreactivity in the GC, CeA, BNST, and LH were identified using sequential scanning with an Olympus confocal microscope. All forebrain areas were identified based on the Paxinos and Watson rat brain atlas (32). The GC and LH are the least defined areas, and a brief description of the location of
retrogradely labeled cells follows. GC was identified as the area approximately between the level at which the anterior commissure crosses midline and the disappearance of the genu of the corpus callosum rostrally. The LH was identified as an area bounded laterally by the optic tract, dorsally by the internal capsule, medially by the fornix, and ventrally by the surface of the brain. The number of immunoreactive cells per section (sum of cells divided by the number of sections) was calculated and used for statistical analyses. Only tissue sections that contained retrogradely labeled neurons were analyzed. Comparisons between forebrain sites were performed using one-way ANOVA and paired-sample t-tests (SPSS 12.0). In some instances, post hoc analyses (Bonferroni) were used to determine the source of statistically significant differences. The results are presented as means ± SE. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Injection sites. In each animal, the taste-responsive region of the PBN was relocated with the FG-filled injection pipette. An example of the response to 0.1 M NaCl applied to the anterior tongue is shown in Fig. 1A. The fluorescent photomicrograph in Fig. 1B shows the resulting FG injection site in the PBN. A brightfield image of the same section is shown in Fig. 1C. Microscopic examination of each injection site revealed that predominantly the central medial, ventral medial, and ventral lateral portions of the caudal PBN were targeted with minimal spread into rostral-lateral regions. Figure 2 shows a summary of the five FG injections into the gustatory responsive PBN.

Retrograde labeling. The number of FG-labeled neuronal cell bodies differed between forebrain sites ($F_{3,163} = 11.8, P < 0.01$). The CeA contained significantly more retrogradely labeled cells compared with the LH, BNST, and GC ($P < 0.01$). The LH, BNST, and GC contained a similar number of FG-labeled cells per section (Fig. 3A; $P = 1.0$). Nevertheless, the number of GC sections ($n = 56$) with retrogradely filled neuronal cells was far greater compared with the LH ($n = 26$) and BNST ($n = 19$) but not the CeA ($n = 59$). Further analyses using paired-sample t-tests revealed a significantly greater total number of FG-labeled neurons in GC ($n = 257$) compared with the LH ($n = 106$; $T = 4.8, P < 0.01$) and BNST ($n = 92$; $T = 2.5, P = 0.03$). The total number of retrogradely labeled cells in the CeA ($n = 608$) was significantly greater than each of the other forebrain areas ($P < 0.05$).

GAD$_{67}$ immunoreactivity. In each forebrain site, immunohistochemical processing for GAD$_{67}$ resulted in robust labeling of cells with distinguishable nuclei and short processes (Fig. 4). Quantification of neuronal cells immunoreactive for GAD$_{67}$ (GAD$_{67}$ ir) in those sections containing FG retrogradely labeled cells is shown in Fig. 3B. Similar to retrogradely labeled cells, the mean number of GAD$_{67}$ ir neurons per section differed between the GC, CeA, BNST, and LH ($F_{3,163} = 32.8, P < 0.01$). GC and BNST contained signifi-

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Fig. 2. Schematic representation of FG injections in the central medial (cm), dorsal medial (dm), ventral medial (vm), and ventral lateral (vl) subdivisions of the PBN. Top to bottom: rostral to caudal. The approximate levels relative to bregma are indicated below each figure (32). Me5, mesencephalic trigeminal nucleus; LPBN, caudal portion of the lateral visceral sensitive PBN; bc, brachium conjunctivum.
The present results also demonstrate that taste-responsive sites within the caudal PBN were targeted to a varying degree by different forebrain areas. The largest source of descending input originated in the CeA followed by GC. The same PBN injections resulted in a considerably smaller number of retrogradely labeled neurons in the BNST and LH.

**Contribution of forebrain areas to descending input to the PBN.** Although each of the forebrain areas examined has been shown to inhibit taste-responsive PBN neurons, the magnitude of inhibition and percentage of neurons under inhibitory control appear to vary with forebrain stimulation site. For instance, Lundy and Norgren (24) showed that more PBN taste neurons were suppressed by activity in the CeA (41/48) and GC (32/45) than by the LH (14/32) in rats. Although the influence of BNST stimulation on PBN taste neurons has not been tested in rats, a recent study in hamsters showed that 98% of PBN taste cells was inhibited by BNST activation (20). These data suggest that the BNST, CeA, and GC are the major sources of neurons with axons projecting to the PBN. This is consistent with the present results showing that the CeA and GC contribute significantly more neurons to descending forebrain-gustatory PBN pathways compared with the LH. In the case of the BNST, however, our results from small physiologically defined injections suggest that the BNST contributes fewer neurons with projections back to the PBN compared with the CeA and GC. It might be that the number of BNST neurons projecting to the PBN differs between hamster and rat or the influence of BNST activation on PBN taste cells is, in part, indirect (e.g., a BNST-CeA-PBN pathway).

**Modulation of PBN gustatory processing.** The predominant inhibition of PBN taste processing by forebrain stimulation suggests a role for the major CNS inhibitory transmitter GABA. Indeed, Kobashi and Bradley (19) have shown that GABA produces a concentration-dependent reduction in input resistance of neurons in the caudomedial gustatory zone of the PBN. Findings from the present double-label experiments, however, demonstrate that forebrain neurons projecting to central medial, ventral medial, and ventral lateral portions of the caudal gustatory PBN do not express GAD_67. This is consistent with a recent electronmicroscopy study investigating the chemical nature of CeA projections to the PBN (18). Although GABA-positive CeA terminals were found to innervate the lateral visceral portion of the PBN, no such terminals were found in the medial gustatory region of the PBN. Together, these data indicate that forebrain-induced inhibition of PBN taste processing is not mediated by direct projections from GABAergic forebrain neurons. One hypothesis is that the visceral-sensitive lateral PBN is a necessary way station for the centrifugal projections that modulate taste processing in the mediod PBN.

Another hypothesis is that some other neurochemical(s) expressed by neurons in the GC, BNST, CeA, and LH mediate descending control of taste processing. For instance, Moga et al. (27) provided evidence for at least five distinct neuropeptide-immunoreactive cell populations in the hypothalamus that project to the PBN. PBN-projecting neurons originating in the CeA (26) and BNST (28) also are immunoreactive for many of these same neurochemicals, including somatostatin (SS), neuropeptide-Y (NPY), corticotrophin-releasing factor (CRF), enkephalin, substance P, and galanin. Following injections of retrogradely labeled neurons in the ventrolateral PBN, cells labeled

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**DISCUSSION**

This study demonstrated that forebrain neurons projecting to taste-responsive sites within the caudal PBN do not contain glutamic acid decarboxylase, the enzyme responsible for the conversion of glutamic acid to GABA. Out of 1,063 retrogradely filled forebrain neurons, only three exhibited GAD_67 immunoreactivity. Prior studies show that activation of GC, BNST, CeA, and LH inhibits PBN neural activity, suggesting a role for the inhibitory neurotransmitter GABA. The present data do not exclude a role for GABA in mediating descending inhibitory control of taste processing but do suggest that it is not due to direct input from GABAergic forebrain neurons.
both for the tracer and NT, SS, or CRF were concentrated in the lateral CeA (26). These investigators mentioned in passing that injections centered within the medial or dorsomedial PBN resulted in labeling confined mainly to the medial CeA but did not present or discuss their neurochemical content. Similarly, for the LH, retrograde tracer injections were aimed at the medial and lateral PBN, but the neurochemical content of LH inputs were not presented or discussed in this context (27). Thus, the relationship between descending forebrain neurochemical pathways and the various sensory inputs processed within the PBN remains to be established. That is, all of these studies used stereotaxic coordinates to place tracer injections, which provided valuable information but did not discern between gustatory, visceral, and somatosensory regions of the PBN.

**Perspectives and Significance**

In the present experiments, we confirmed and extended prior data that examined the neurochemical content of forebrain neurons projecting to the gustatory PBN. Considerable evidence suggests that the reciprocal connections between the PBN and ventral forebrain are critical for elaborating and probably for altering the hedonic value of a taste. If we assume that the influence of centrifugal input is mediated by direct projections to the gustatory region of the PBN, then GABAergic forebrain neurons apparently are not part of this descending pathway. In terms of taste response inhibition, one alternative is that intrinsic GABAergic neurons interface between descending axon terminals and PBN gustatory neurons. This synaptic arrangement has been hypothesized to mediate the inhibitory influence of GC on second-order taste neurons in the nucleus of the solitary tract (37). These cells are maintained under tonic GABAergic inhibition, and GC-induced inhibition is blocked by local application of the GABA<sub>A</sub> receptor antagonist bicuculline. Alternatively, centrifugal influences might be mediated by an indirect pathway to PBN taste cells that first synapses in the lateral visceral sensitive region of the PBN. The PBN is a critical substrate for the integration of gustatory and more rostrolaterally processed visceral information, and physiological factors associated with ingestion modulate PBN taste responses (4, 15). GABA-positive CeA terminals innervating the lateral PBN have been reported (18). Future studies using electrophysiological, anatomical, and lesion-behavioral techniques will continue our efforts to determine the neurochemicals and neural circuit(s) that mediate centrifugal control of taste processing in the brain stem.

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**GRANTS**

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