Expression of Fos during sham sucrose intake in rats with central gustatory lesions

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Mungarndeey SS, Lundy RF Jr, Norgren R. Expression of Fos during sham sucrose intake in rats with central gustatory lesions. Am J Physiol Regul Integr Comp Physiol 295: R751–R763, 2008.—For humans and rodents, ingesting sucrose is rewarding. This experiment tested the prediction that the neural activity produced by sapid sucrose reaches reward systems via projections from the pons through the limbic system. Gastric cannulas drained ingested fluid before absorption. For 10 days, the rats alternated an hour of this sham ingestion between sucrose and water. On the final test day, half of them drank water and the other half 0.6 M sucrose. Thirty minutes later, the rats were killed and their brains immunohistochemically stained for Fos. The groups consisted of controls and rats with excitotoxic lesions in the gustatory thalamus (TTA), the medial (gustatory) parabrachial nucleus (PBN), or the lateral (visceral afferent) parabrachial nucleus.

In controls, compared with water, sham ingesting sucrose produced significantly more Fos-positive neurons in the nucleus of the solitary tract, PBN, TTA, and gustatory cortex (GC). In the ventral forebrain, sucrose sham licking increased Fos in the bed nucleus of the stria terminalis, central nucleus of amygdala, and the shell of nucleus accumbens. Thalamic lesions blocked the sucrose effect in GC but not in the ventral forebrain. After lateral PBN lesions, the Fos distributions produced by distilled H2O or sucrose intake did not differ from controls. Bilateral medial PBN damage, however, eliminated the sucrose-induced Fos increase not only in the TTA and GC but also in the ventral forebrain. Thus ventral forebrain areas associated with appetitive responses appear to be activated directly by PBN gustatory neurons rather than via the thalamocortical taste system.

SUCROSE IS NORMALLY REWARDING to rats and many other animals. The neural bases for this hedonic effect are not understood, but, at some point, the afferent neural activity must engage central reward systems. Sucrose ingestion releases dopamine (DA) in the nucleus accumbens (16). This DA release is concentration dependent but is independent of the volume consumed or the nutritional consequences of the ingestion (19). For these and other reasons, accumbens DA release can be taken as a central index of the reward value of the gustatory stimulus (40). From this, it follows that understanding how gustatory afferent activity reaches forebrain reward systems will aid in detecting the neural underpinnings of hedonic value.

In the rat, the central gustatory system bifurcates in the pons into a dorsal, thalamocortical projection and a ventral, limbic distribution (30). Lesions of the thalamic taste relay have no effect on accumbens DA release during sucrose licking. The same DA response is substantially blocked, however, when the lesions are in the pontine parabrachial nuclei (PBN), the second central gustatory relay (18). This implies that the limbic gustatory projection originating in the PBN supports this DA index of reward but that the thalamocortical taste system contributes little if anything to the process.

This prior experiment revealed which of the central gustatory projections was responsible for the accumbens DA response during sucrose ingestion, but the data did not provide any further clues about the neural systems involved. The experiment summarized in this report uses a similar paradigm, i.e., damaging central relays to determine from what level gustatory afferent activity influences forebrain systems. The dependent variable, immunohistochemical labeling of Fos, differs from microdialysis in that it provides information about increased neural activity in the entire brain rather than a limited area adjacent to the dialysis probe. Thus the current experiment extends the DA dialysis data by providing clues as to where central gustatory activity is processed before it reaches nucleus accumbens. Several other studies have examined the distribution of the protein products of the immediate early gene fos during sapid sucrose stimulation, but none have done so using sham intake or central lesions (9, 15, 47, 70).

MATERIALS AND METHODS

Subjects

Twenty-eight male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing between 300 and 350 g, were housed individually in hanging wire mesh cages on a 12:12-h light-dark cycle (lights on at 0700) in a temperature and humidity controlled vivarium. The animals were maintained on a standard laboratory diet (Rodent Diet-W 8604; Harlan Teklad, Madison, WI). All manipulations occurred during the light phase. The procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and comply with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

Surgical Procedures

Excitotoxic brain lesions. The rats were divided into groups defined by the target of their bilateral lesions, i.e., the thalamic taste area (TTA; n = 6), medial PBN (mPBN; n = 6), lateral PBN (lPBN; n = 6), and controls (Con; n = 10). The latter rats received treatment identical to the experimental animals except that they had bilateral infusions of 0.1 M phosphate-buffered saline (PBS) instead of ibo-

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tenic acid (Ibo; Research Biochemicals International, Natick, MA) in the medial PBN or thalamic taste area (n = 3 each). The remaining four rats served as unoperated (naive) controls (UnOp; n = 4).

Because each group was divided into rats lacking sucrose and other rats lacking water on the test day, the sample size n for each group condition was small: three for the lesioned groups and five for controls. This was a compromise dictated by the necessities of design and the time required to complete the experiment. Small samples increase the likelihood of type I and II errors. We ameliorated the risk of type I errors by using a conservative post hoc test (Newman-Keuls) after obtaining significant ANOVA values.

The rats were food deprived overnight, injected with atropine (0.1 mg ip; Genesia Sicor Pharmaceuticals, Irvine, CA) and gentamicin (4 mg ip; Abbott Laboratories, North Chicago, IL) 15 min before being anesthetized with pentobarbital sodium (50 mg/kg ip; Nembutal; Abbott Laboratories, North Chicago, IL), and mounted in a stereotaxic apparatus equipped with nontraumatic ear bars (David Kopf Instruments, Tujunga, CA). Supplemental anesthetic doses of pentobarbital sodium (15 mg/kg) were given as needed. The subsequent procedures closely followed published routines and, thus, their descriptions are abbreviated (14, 52, 56).

Under aseptic conditions, the skull was exposed with a midline incision and leveled between β and λ. Bilateral holes were drilled dorsal to the intended target areas. For the TTA, the holes were centered at −3.5 mm from β (AP), laterally 1.2 mm from midline (ML); for both PBN lesions, the holes were −12.0 and 1.8 mm, respectively. Both the thalamic and pontine gustatory areas were located first by monitoring multiunit activity with a glass-insulated tungsten microelectrode [impedance (Z) = 1.0–1.5 MΩ at 1 kHz] using conventional amplification techniques. Once the electrode was located, the posterior tip was stimulated with 0.3 M NaCl and deionized, distilled water (dH2O). Particularly in the TTA, cool dH2O (10–15°C) and warm dH2O (30–38°C) also were used as stimuli. For the PBN, the electrodes were tilted 20° off perpendicular (with the tip anterior) to avoid the transverse sinus. For the TTA, the electrodes were vertical.

Once a taste response was located bilaterally, typically about five penetrations, the recording electrode was replaced with a double-barreled glass micropipette (outer diameter 50 – 60 μm; Z = 0.5–1.0 MΩ at 1 kHz). One barrel was glued onto the needle of a 1-μl Hamilton microsyringe filled with mineral oil (Hamilton, Reno, NV); the other contained an etched tungsten wire. Ibo (20 μg/μl in 0.1 M PBS, pH 7.2) was drawn up into the pipette tip immediately before the injections. This pipette assembly was then repositioned to the appropriate coordinates, and for the TTA and mPBN, the gustatory responses were checked by recording through the microelectrode pipette during rapid stimulation. For the IPBN lesions, the infusion pipette was moved 0.5 mm anterior, 0.5 mm lateral, and 0.5 mm ventral to the coordinates for the best taste response. Once relocated, 150 nl of Ibo were infused over 10 min, and the pipette was left in place for an additional 10 min. The sham-operated rats were treated identically except that the Ibo was replaced with 150 nl of PBS (pH 7.2). After the bilateral infusion, the skull openings were filled with Gelfoam (Upjohn, Kalamazoo, MI) and the skin was closed with wound clips. The animals typically recovered their presurgical body weight within a week.

**Gastric fistula surgery.** After 1 wk, the rats were fitted with a stainless steel gastric cannula (13 × 6-mm inner diameter) as described previously (19, 62). Briefly, the rats were food deprived overnight and anesthetized as described previously. The stomach was exposed via a midline incision, a second small incision was made in the muscular wall of the greater curvature, and the flange at the base of cannula (16-mm outer diameter) threaded into this hole. The hole was then closed with a purse string suture (surgical silk 3/0; Ethicon, Somerville, NJ). A donut of Marlex mesh (Baird, Murray Hill, NJ) was placed over the shaft of the cannula and fitted snugly against the stomach. The shaft of the cannula was pulled through a stab incision in the abdomen and held in place with a threaded stainless steel washer. The peritoneum was sutured with interrupted stitches, erythromycin ophthalmic ointment was applied (0.5%; Bausch & Lomb, Tampa, FL), and the original midline incision was closed with wound clips.

**Training Protocol.**

Because of the logistics of the histology, the rats were run in seven squads of four each. The squads were balanced across the experimental groups as closely as possible, i.e., the three groups with lesions had an n of 6 each, but there were 10 controls. Before and after surgery, each squad was put on a deprivation schedule with food and water available for 2 h in the afternoon. For 2 wk after gastric surgery, however, food and water were available ad libitum. When the deprivation schedule was reinstalled, the rats had access to water for 15 min in the morning (0900) and 2 h in the afternoon (1400–1600), both times with the gastric cannula closed, i.e., real intake. At this point, food intake also was restricted to the 2-h afternoon slot for the duration of the experiment (Fig. 1).

Over the next 7 days, the morning session was changed to 60 min of sham drinking to expose rats to the fluid stimuli long enough to generate sufficient Fos protein (adaptation, days 8–14). Before these daily sessions, the gastric cannulas were opened and the stomach contents removed by repeated flushing with lukewarm water (~20 ml; Ref. 62). Each rat then was presented with a water spout in a hanging plastic cage (25.0 cm long × 28.5 cm wide × 35.0 cm high) with a

<table>
<thead>
<tr>
<th>Session</th>
<th>Deprivation Training</th>
<th>AM Sham Intake</th>
<th>Alternate water and sucrose</th>
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<tbody>
<tr>
<td>Days</td>
<td></td>
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<td>Homcage</td>
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<td>16  17  18  19</td>
<td>20  21  22  23  24*</td>
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<td>Shm drk cage</td>
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<tr>
<td>Gastric fistula</td>
<td>Closed</td>
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* Day 24 = Test day and perfusion for FOS immunohistochemistry; * W = water; * S = 0.6 M sucrose;

Fig. 1. Summary of behavioral sessions.

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slot down the center of the wire floor to accommodate the gastric cannula drain tube. At the end of the sham intake session, the stomach was again flushed with warm water to minimize nutrient absorption, the fistula was closed, and the animals were returned to their home cages. The sham drinking effluent was collected in plastic containers below the test cages and measured. The volume of drainage collected had to meet or exceed the fluid consumed, or the data were discarded. Sham intake typically stabilized within 7 days (8). The afternoon feeding and real drinking schedule remained the same.

The experimental period began when two of the four rats in a squad were switched to 0.6 M sucrose (Fisher Chemicals, Fairlawn, NJ) for the morning sham intake session. The other two rats continued with dH2O. The next morning, all four rats were switched from sucrose to dH2O or from dH2O to sucrose. This alternating pattern continued for 10 days (alternate drinking; days 15–24, see Fig. 1). This was sufficient to provide stable fluid intake regardless of the stimulus. On day 24, 30 min after the end of the sham intake session, the four rats were killed and processed for immunohistochemistry.

**Immunohistochemistry Procedures**

After the final sham intake session, the rats were deeply anesthetized (pentobarbital sodium, 100 mg/kg ip) and perfused transcardially for 5 min with 300 ml of cold, heparinized physiological NaCl (1.5:1,000), followed by 300 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were then postfixed for an hour in situ. Once removed from the skull, they were immersed in 20% sucrose in 4% paraformaldehyde for another 2–3 h and then cryoprotected in 20% sucrose-0.1 M PB overnight at 4°C. Subsequently, the brains were sectioned coronally at 50 μm on a freezing microtome, and the sections were collected in three series. The first series was processed for Fos immunohistochemistry; the second was processed for a neuron-specific protein, NeuN (22, 33); and the third was stored in a cryoprotectant solution for a replication if one of the first two series was inadequate. Sections from control brains were processed in tandem with experimental brains to minimize immunohistochemical variability.

Fos immunohistochemistry. After being rinsed with 0.1 M PB, the sections were treated with 1% NaBH4 (Sigma-Aldrich, St. Louis, MO) in 0.1 M PBS (pH 7.3–7.4) for 20 min at room temperature (RT) to remove endogenous peroxidase, rinsed in PBS several times, and then incubated 36–48 h at 4°C in primary polyclonal IgG rabbit Fos antibody diluted in 0.4% Triton X-100/PBS (1:10,000; catalog no. SC-52; Santa Cruz Biotechnology, Santa Cruz, CA). After further rinses with PBS (4 × 15 min), the sections were incubated with secondary biotinylated goat anti-rabbit IgG in 0.4% Triton X-100 (1:500; catalog no. 62-6140; Zymed Laboratories, San Francisco, CA) mixed with goat serum (catalog no. G-6767; Sigma Chemical) for 2 h at RT. After four more PBS rinses, the sections were incubated in conjugated avidin-biotin complex (1:200; Vectastain Elite ABC reagent, catalog no. PK-6100; Vector Laboratories, Burlingame, CA) in 0.4% Triton X-100/PBS for 1 h at RT and then rinsed again four times for 15 min each in PBS. Subsequently, a visible immunoreactivity product was produced after 5 min of incubation in 69 ml of 0.175 M sodium acetate for 5 min. The reaction was monitored visually and stopped with rinses of 0.1 M PBS.

Image Acquisition and Statistical Analysis

Fos distribution and quantification. The sections were examined with a light microscope (Nikon Optiphot, Tokyo, Japan) equipped with a digital video camera (Diagnostic Instruments, Sterling Heights, MI). With the objective focused on the upper surface of the section, specific regions were captured with video-imaging software (Spot for Windows, version 4.0.4) and subsequently analyzed with Optimas (Bioscan, Edmonds, WA). The areas of interest were defined based on the adjacent NeuN series and appropriate atlas plates (46). Within these areas, the Fos immunoreactivity profiles were counted by Optimas based on a grayscale reference. Whenever possible, the same grayscale threshold was maintained for all areas within each brain. In any event, counting was not automatic. The program outlines each counted profile, and this permitted the investigator to inspect it both in the digitized image and through the microscope. This reduced counting artifacts and undercounting labeled nuclei that are stacked on one another. Most areas require counting over multiple sections, but the same number of sections was counted for each area in all brains. The results are presented as the average number of labeled profiles per area per rat. The statistics were done using the average counts per area across rats. The same 13 nuclear areas were counted in all brains (Table 1). This semiautomated procedure was checked for 10 areas by using a 25% sample of “by-eye” counts made by individuals blinded to the experimental protocol. The correlation (r) between the two methods averaged +0.87 (P < 0.001).

**Lesion identification.** The adequacy of the lesions was judged by comparing the areas without neurons with comparable areas in the

<table>
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<th>Areas of Fos Counts</th>
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<tr>
<td>Central gustatory areas</td>
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<tr>
<td>Nucleus of solitary tract, rostral portion</td>
</tr>
<tr>
<td>Medial parabrachial nucleus, gustatory</td>
</tr>
<tr>
<td>Lateral parabrachial nucleus, visceral</td>
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<tr>
<td>Thalamic taste area, parvicellular portion of ventroposteromedial nucleus</td>
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<tr>
<td>Gustatory cortex, dysgranular insular cortex</td>
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<tr>
<td>Fluid balance areas</td>
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<tr>
<td>Subfornical organ</td>
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<tr>
<td>Supraoptic nucleus</td>
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<tr>
<td>Paraventricular nucleus</td>
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<tr>
<td>Limbic and dopamine areas</td>
</tr>
<tr>
<td>Nucleus accumbens, shell subnuclei</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Central nucleus of amygdala</td>
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<tr>
<td>Lateral hypothalamus</td>
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<tr>
<td>Ventral tegmental area, area 10</td>
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Fos-labeled neurons were counted in 13 areas divided into 3 functionally related groups: central gustatory, fluid balance, and limbic and dopamine areas.
brains of the PBS-injected controls. The gustatory areas of the mPBN and TTA were defined from neuroanatomical and electrophysiological data from prior published studies in this laboratory (26, 27, 38, 43, 56).

**Statistical analysis.** Repeated-measures ANOVA were performed on the behavioral data from each stage of training. Similarly, ANOVA were performed to compare the number of Fos-positive neurons in each brain region across stimuli (1 factor) and across lesions (lesions × stimuli, 2 factors). In both analyses, significant main effects or interactions were followed by post hoc comparisons between treatment means using the nonparametric version of the Newman-Keuls test (Statistica version 7.1, edition 2005; StatSoft, Tulsa, OK). The level of significance (α) was set at 0.05. In text, NS indicates not significant.

**RESULTS**

**Lesions**

There were three types of lesions, those centered on the mPBN, the IPBN, and the TTA. Each group had six rats, three of which sham licked sucrose on the last day and three that licked water. The thalamic lesions were the most consistent in that the taste area was destroyed bilaterally in each of the six rats (Fig. 2, A and B). All the lesions joined across the midline and, in doing so, damaged both the retroreuniens and center median nuclei. Laterally, they spread into the adjacent oral trigeminal relay, and dorsally, they invaded the intralaminar and mediodorsal nuclei to varying degrees. In the pons, the analysis was more complex, because the lesions were intended to be functionally rather than anatomically specific. The mPBN lesions were centered electrophysiologically on gustatory responses, but the resulting neuronal damage extended laterally both dorsal and ventral to the brachium conjunctivum (BC; Fig. 2, C and D). Ventrally, taste neurons extend to the lateral tip of BC, but dorsally, they occur only over the medial one-third to one-half of the tract (43). Thus, in all save one of the rats, neurons were destroyed in at least part of the posterolateral PBN in which neurons probably do not respond to sapid stimuli. Similarly, the other set of PBN lesions were centered laterally, ventrally, and anteriorly to the medial ones, but they were large enough that most spread into the taste areas (Fig. 2, E and F). Nevertheless, of the six IPBNx rats, the lesions in five spared some medial, presumably taste neurons on at least one side. The PBN lesions usually also spread into adjacent areas, primarily the locus coeruleus medially and the supratrigeminal area ventrally.

**Fig. 2.** Digitized photomicrographs of NeuN-stained coronal sections through the thalamic and pontine gustatory areas. A: control thalamus with gustatory-lingual area outlined [arrows, rat 02559, adapted from Mungrande et al. (34)]. B: thalamic lesion centered on gustatory relay (rat 03173). C and D: left and right side of medial parabrachial nucleus (PBN) lesions centered on gustatory responses but extending into posterior lateral PBN (IPBN; rat 03163). E and F: IPBN lesions showing minimal (left, rat 03166) and substantial (right, rat 03168) sparing of the medial, gustatory PBN (arrows at right). BC, brachium conjunctivum; CM, central medial thalamic nucleus; Hb, habenula; LC, locus coeruleus; ml, medial lemniscus; MoV, trigeminal motor nucleus; OPC, oval paracentral nucleus; PF, parafascicular thalamic nucleus; Ra, retroreuniens area; TTA, thalamic taste area; 3V, third ventricle. Scale bars in A (for A and B) and E (for C–F) = 0.5 mm.
Behavior

For a week (days 1–7), all the rats were trained to real drink distilled water for 15 min in the morning and 1 h in the afternoon. During the second week (days 8–14), they were adapted to overnight food and water deprivation, to sham drinking water for 1 h in the morning, and to real drinking and eating for 2 h in the afternoon. While maintained on the same overnight deprivation schedule, they then sham drank water or 0.6 M sucrose alternately for 10 days. Half the rats began with water, and the other half with sucrose. Thirty minutes after the last sham intake session, the rats were killed and their brains processed for Fos immunoreactivity. At each stage, we analyzed the intake data from the two control groups first (UnOp vs. PBS; n = 4 and 6, respectively) and then combined them (Con) for comparison with the data from the three experimental groups (mPBNx, IPBNx, and TTAx).

The PBS and UnOp control groups were combined because across the 24 days of testing, they did not differ consistently in body weight, food consumption, or morning and afternoon water intake. During the last period, when they alternated sham drinking water and sucrose, the UnOp rats ingested significantly more sucrose than the PBS group. Expressed as a function of body weight (ml/100 g), however, much of that difference disappeared, and what remained was no longer statistically significant [PBS, 11.0 ± 1.05 ml vs. UnOp, 12.9 ± 1.22 ml; F(1.8) = 3.16, P = 0.11, NS]. To simplify the behavioral results, the analysis of these two control subgroups is not presented in further detail.

Training. Over the week of training, the mPBNx group weighed an average of 508.3 g (± 18.6 g SE), which was ~110 g or 18% lighter than the Con rats (619.0 ± 20.7 g), but the IPBNx (590.3 ± 21.6 g) and TTAx (621.1 ± 21.8 g) groups were statistically equivalent to the controls [F(3,24) = 5.79, P = 0.04; post hoc Con vs. mPBNx, P = 0.004]. Overall, afternoon water consumption was lower for the three experimental groups [F(3,24) = 5.59, P = 0.005], but only the IPBNx rats were down significantly (Con, 7.2 ± 1.5 ml; mPBNx, 3.3 ± 0.7 ml; lPBNx, 4.3 ± 0.7 ml; TTAx, 5.7 ± 1.6 ml; F(1.8) = 3.16, P = 0.11, NS). Across the week, the mPBNx group lost weight significantly more than the Con group [Con, 7.2 ± 1.5 ml; mPBNx, 14.9 ± 2.2 ml; lPBNx, 12.8 ± 2.1 ml; F(1.8) = 6.8 ml, P = 0.004; post hoc Con vs. mPBNx, P = 0.003], but none of the post hoc comparisons was significant (Con, 7.2 ± 1.5 ml; mPBNx, 14.9 ± 2.2 ml; lPBNx, 12.8 ± 2.1 ml; TTAx, 14.7 ± 1.8 ml; F(1.8) = 6.8 ml, P = 0.004; others NS). Overall average weight drifted down 24 g (4.5%) over these 10 days [F(9,216) = 20.89, P < 0.001], and because of the large difference between the mPBNx group and the others, the interaction also was significant [F(27,216) = 2.07, P = 0.002]. Although the mPBNx rats weighed almost 20% less than the Con animals, they ate the same amount [F(3,24) = 1.12, P = 0.36, NS]. Average water intake was down 24 g (4.5%) over these 10 days [F(9,216) = 20.89, P < 0.001], and because of the large difference between the mPBNx group and the others, the interaction also was significant [F(27,216) = 2.07, P = 0.002]. Although the mPBNx rats weighed almost 20% less than the Con animals, they ate the same amount [F(3,24) = 1.12, P = 0.36, NS]. The average intake did vary across the period [F(8,192) = 3.03, P = 0.003] but not systematically across the four groups [F(24,192) = 1.48, P = 0.08, NS]. Afternoon water intake, on the other hand, mirrored the weight differences in that the mPBNx rats drank less than controls, but the IPBNx and TTAx groups did not [Con, 16.8 ± 2.2 ml; mPBNx,
Afternoon food intake during the 10-day period of alternating AM fluids

The most relevant behavioral comparisons are within groups between sham water and sucrose on the last day (Fig. 4, A and B, far right enclosures), because they directly parallel the most meaningful differences in the Fos data. A separate ANOVA of these data produced significant main effects across groups [F(3,20) = 11.29, P = 0.0002] and stimuli [F(1,20) = 10.57, P = 0.0035] but no interaction [F(3,20) = 0.78, P = 0.52, NS]. Thus, on the last day of testing, there were no significant differences between sham water and sham sucrose intake within groups.

**Behavior summary.** Compared with the combined controls, the mPBNx rats weighed less but ate similar amounts. The IPBNx and TTAX groups did not differ from the Con groups on either measure. Afternoon water intake for the lesion groups differed from Con only twice in nine possible comparisons, the

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**Table 2. Afternoon food intake during the 10-day period of alternating AM fluids**

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<th>2-h PM Food Intake, g</th>
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<tr>
<td></td>
<td>Day 16</td>
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<tr>
<td><strong>Water test squad</strong></td>
<td></td>
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<tr>
<td>Con</td>
<td>536.2±23.5</td>
</tr>
<tr>
<td>mPBNx</td>
<td>444.0±30.7</td>
</tr>
<tr>
<td>IPBNx</td>
<td>533.7±22.9</td>
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<tr>
<td>TTAX</td>
<td>574.3±3.4</td>
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<tr>
<td><strong>Sucrose test squad</strong></td>
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<tr>
<td>Con</td>
<td>531.4±27.3</td>
</tr>
<tr>
<td>mPBNx</td>
<td>434.0±44.8</td>
</tr>
<tr>
<td>IPBNx</td>
<td>489.0±21.4</td>
</tr>
<tr>
<td>TTAX</td>
<td>517.3±20.1</td>
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Rats were divided first based on their lesions: medial parabrachial nucleus (mPBNx; n = 6), lateral parabrachial nucleus (IPBNx; n = 6), thalamic taste area (TTAX; n = 6), and the combined controls (Con; n = 10). These groups were subdivided equally into 2 squads: one that sham ingested distilled water on the last test day and the other that received 0.6 M sucrose; the order was counterbalanced. Food intake values are means ± SE for the days indicated; body weight values are means ± SE for the test day (day 24). No significant differences existed between the surgical (n = 6) and unoperated (n = 4) controls in body weight, food intake, or water drinking, so the groups were merged into one Con group. (See RESULTS for further details.)
mPBNx and iPBNx rats once each. For both the training and adaptation periods, the main effect of morning water intake was significant across groups, but none of the post hoc comparisons between the lesion groups and the Con contributed to that difference. During the alternating phase, the rats sham drank more sucrose than water, but the pattern across groups was similar for both stimuli, i.e., mPBNx rats licked less than the Con group, and the iPBNx and TTAx groups took more. On the last day (day 24), within groups there were no significant differences between water and sucrose sham intake.

Fos Immunohistochemistry

The 13 areas analyzed for Fos immunoreactivity are grouped by function, those related to taste, fluid balance, and motivation or reward. Because taste was an independent variable, i.e., water vs. sucrose, the analysis of central areas related to taste tested the validity of the experimental design. Similarly, because all the rats were water deprived overnight, Fos expression was counted in areas related to fluid balance to test whether thirst was a differential factor. Food intake also was restricted to regularize the degree of hunger, because water deprivation induces hypophagia (58, 68). Finally, the limbic and DA-related areas were included to determine whether sucrose taste alone would produce differential activation.

The Fos-positive cells in all 13 areas were compared across the two control subgroups, PBS (n = 6) and UnOp (n = 4). Compared with water, sham intake of sucrose produced more Fos in many areas [F(1,6) = 22.66, P = 0.003], but there was no influence of the control subgroups [F(1,6) = 1.81, P = 0.23, NS]. Overall, the interaction comparing these 2 subgroups and stimuli across all 13 areas was not significant [F(12,72) = 0.45, P = 0.94, NS].

Gustatory nuclei. Baseline differences in Fos are summarized in the Control (Con) values (figure 5, column 1) and for the rostral nucleus of the solitary tract (rNST; Row 5) in Fig. 5. Illustrative brain sections from which these data were derived appear in Fig. 6. In the Con rats, sham intake of 0.6 M sucrose resulted in significantly more Fos label than similar intake of water at all levels of the central gustatory system from the medulla to the cortex [F(1,8) = 33.95, P = 0.0004; Fig. 5, Con]. Similarly, in the first central gustatory relay, the rNST, sham licking sucrose produced more Fos-positive neurons than water regardless of where lesions were placed in the brain (taste: F(1,20) = 292.52, P < 0.001; lesions: F(3,20) = 2.28, P = 0.11, NS; interaction: F(3,20) = 1.68, P = 0.20, NS; Fig. 5, rNST). Figure 5 also documents the effects of bilateral lesions centered on gustatory neurons in the mPBN, the second central taste relay in rats [F(3,12) = 14.01, P < 0.001; Refs. 38, 41].

In the rNST, distal to the lesion, sucrose licking produced a robust increase in Fos (post hoc P = 0.007). Central to the mPBN damage in the thalamic (TTA) and cortical (GC) taste areas, however, sham intake of sucrose elicited Fos expression equivalent to that of water (post hoc P = 0.96 for TTA and 0.07 for GC). Lesions one synapse further central in the TTA blocked the sucrose effect on cortex but left the differential Fos expression in both the medulla and pons [F(3,12) = 38.11, P = 0.001; post hoc water vs. sucrose, P = 0.46 for GC, 0.003 for rNST, and 0.016 for mPBN; Fig. 5, TTAx]. Lesions in the IPBN, which contains neurons of a parallel visceral afferent system (39, 54), failed to block the increased Fos produced by sham sucrose intake both in the medulla and on cortex [F(3,12) = 10.95, P = 0.0009; post hoc water vs. sucrose, P = 0.04 for rNST and 0.011 for GC; Fig. 5, IPBNx]. Lesions in the mPBN and IPBN overlapped considerably, so comparing Fos label in their parabrachial neighbor was not meaningful (Fig. 5, indicated by X). These results established that the lesions have the effect predicted by the anatomy of the central gustatory system. Peripheral to the damage, sham sucrose intake increases Fos activity as it does in controls; central to it, the same taste stimulation has no more influence on Fos production than water.

Fluid balance areas. Of the three areas known to be involved in fluid balance, the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) exhibited significant differences in Fos label as a function of sham ingesting water or sucrose, but the central lesions had no effect, i.e., the main effect of lesions and the interaction term were not significant (Table 3; Fig. 7). Despite this lack of significance, it was noted that for the SON and PVN, the biggest differences across groups were in the Fos counts from rats sham drinking water rather than sucrose (Fig. 7). This observation is consistent with the role of these areas in fluid imbalances.
Limbic and dopaminergic nuclei. Compared with water, control rats sham licking sucrose produced significantly more Fos-positive neurons in the bed nucleus of the stria terminalis (BNST), the central nucleus of the amygdala (CNA), and the nucleus accumbens shell (NAcS), but not in either the lateral hypothalamus (LH) or the ventral posteromedial thalamic nucleus (VTA; (stimuli \times nuclei) interaction: $F(4,32) = 5.84, P = 0.0012$; post hoc sucrose vs. water, $P = 0.05$ for BNST and NAcS, $P = 0.01$ for CNA, and NS for LH and VTA; Fig. 8, Con). The pattern in the TTAx and lPBNx groups (Fig. 8) was nearly identical (TTA: interaction $F(4,16) = 24.35, P = 0.001$, post hoc sucrose vs. water, $P < 0.05$ for BNST and CAN, and NS for NAcS, LH, and VTA; lPBN: interaction $F(4,16) = 7.64, P = 0.0012$, post hoc sucrose vs. water, $P < 0.05$ for BNST and CAN, and NS for NAcS, LH, and VTA). Bilateral lesions in the mPBN (Fig. 8), however, eliminated the effects of sham sucrose licking in BNST, CNA, and NAcS but did not alter the distributions in LH or VTA [interaction: $F(4,16) = 0.70, P = 0.60$, NS]. Figure 9 shows photomicrographs of representative forebrain area from which these data were derived.

**DISCUSSION**

Compared with water, sham licking 0.6 M sucrose for an hour elicited more Fos protein production in each of the central gustatory nuclei. To underscore the classic concept of a sensory relay, lesions of the intermediate gustatory relays in the pons and thalamus eliminated the effect of taste stimuli on Fos in gustatory cortex but not in the medulla. In rodents, at least, gustatory afferent activity also reaches the forebrain via a separate pathway that arises in the medial parabrachial nuclei, the second central relay, and distributes widely in the limbic system. In two of these limbic targets, the CNA and the BNST, sham licking sucrose also increased Fos compared with water. The same sucrose effect appeared in the NAcS but was absent in the LH and the VTA. The thalamic lesions that block the sucrose Fos effect in gustatory cortex spare it in the BNST, CNA, and NAcS. Medial parabrachial lesions, however, block both the thalamocortical sham sucrose increases and those in the ventral forebrain.

As an index of neural activity, immunohistochemical labeling of Fos has the advantage of compatibility with complex

**Table 3. Statistical analysis of Fos-labeled neurons in the fluid balance areas**

<table>
<thead>
<tr>
<th>Fluid Balance Nuclei</th>
<th>Statistics of 2-Way ANOVA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Taste (Main effect)</td>
<td>Lesions (Main effect)</td>
<td>Taste × lesions (Interaction)</td>
</tr>
<tr>
<td>Subfornical organs</td>
<td>$F(1.20) = 0.04$</td>
<td>$F(3.20) = 1.02$</td>
<td>$F(3.20) = 0.14$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.84$, NS</td>
<td>$P = 0.40$, NS</td>
<td>$P = 0.94$, NS</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>$F(1.20) = 4.53$</td>
<td>$F(3.20) = 0.78$</td>
<td>$F(3.20) = 0.04$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.046$</td>
<td>$P = 0.52$, NS</td>
<td>$P = 0.99$, NS</td>
</tr>
<tr>
<td>Paraventricular nucleus of hypothalamus</td>
<td>$F(1.16) = 12.65$</td>
<td>$F(3.20) = 0.74$</td>
<td>$F(3.20) = 0.99$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.003$</td>
<td>$P = 0.50$, NS</td>
<td>$P = 0.42$, NS</td>
</tr>
</tbody>
</table>

NS, not significant.
behavior in freely moving animals. Often as not, however, this advantage also becomes the undoing of the resulting data. The technique is indiscriminate; if repeated often enough, anything that raises neural activity can increase Fos. In this experiment, we attempted to control for as many of these extraneous variables as possible. The fluid stimuli differed on one major dimension: taste. [The viscosity of 0.6 M sucrose also is somewhat greater than that of water.] The rats were sham fed to reduce visceral afferent and metabolic feedback to a minimum. They had considerable and equal experience with ingesting water and sucrose to eliminate novelty. They were both water and food deprived. Water deprivation drives the ingestive behavior but also decreases food intake (68). The food deprivation equalized that factor across rats but may have increased hunger above that produced by the dehydration alone.

Regardless of the cause, on average, the rats sham ingested more sucrose than water, but those differences within groups were not as large as the intake differences among groups (Fig. 3, A and B). In fact, the goal was to vary reward, not intake. Clamping the sucrose intake to that of water would have controlled for volume. Under those conditions, however, it is likely that the rats would have finished their ration of sucrose before the 60-min trial was over. Because it is unclear how this would influence the reward value of the sucrose, we chose to permit ad libitum intake.

Although the current objective was to follow gustatory afferent activity related to reward, the possibility remains that the Fos increases resulted from the volume differences rather than reward per se. To address this issue, if only indirectly, we included three nuclei known to be involved in fluid balance in the Fos counts. In these areas, the number of Fos-positive profiles did not vary consistently as a function of the fluid ingested. The possibility that the volume ingested contributed to the Fos differences was more directly, if inadvertently, dealt with in a pilot study in which normal rats sham drank either water or 0.2 M sucrose. Other than the concentration of the sucrose, the experimental circumstances were identical to those in the current study. To our chagrin, the immunohistochemical data produced no differences in Fos-positive neurons between the brains of rats licking sucrose or water. Nevertheless, in their final 60-min bouts, the sucrose rats ingested an average of 30.0 ml more fluid than the water rats. In the current experiment, the control rats licking 0.6 M sucrose ingested 28.4 ml more than those that had water. Thus volume differences alone are unlikely to account for the substantial differences in Fos labeling in the current water and sucrose groups.

If volume was not critical, then other dimensions of the fluid stimuli such as taste and reward value become more plausible factors to account for the differences in Fos counts. The central gustatory nuclei were included specifically because they have well-documented excitatory connections from the taste buds on
the tongue and oral cavity (30). The fact that the central taste relays contained more Fos in control rats sham ingesting sucrose than in those licking water demonstrates that peripheral gustatory neural activity can induce the c-fos gene across four synapses. This is far from a novel observation. Electrophysiological experiments in anesthetized animals have reported gustatory responses in the thalamus and even on cortex for decades (27, 28, 29). The Fos technique also has been used previously to assess central gustatory activity in both the sensory relay nuclei and elsewhere, although seldom during sham feeding or after central lesions (9, 25, 31, 72). Regardless, in the current study, these data were important benchmarks for evaluating the Fos effects in other nuclei. The central lesions were placed in the two intermediate central gustatory relays in the pons and the thalamus. In these groups, the difference in Fos in the NST, the first central taste relay, indicated that ingesting sucrose remained an effective stimulus compared with water. In gustatory cortex, the absence of a difference in Fos indicated that the lesions were effective in blocking the gustatory afferent activity from reaching more central relays. This juxtaposition in turn was critical for evaluating the parallel effects of the stimuli and lesions in ventral forebrain areas that are not known for robust or specific gustatory responses but are linked to motivation and reward (1, 23, 35, 36, 44).

The CNA and BNST influence a variety of motivated behaviors, including feeding (5, 45, 67), and receive direct gustatory projections from the PBN (38). Lesions of the TTA block the Fos increases in gustatory cortex after sham sucrose intake but have no effect on similar increases in the CNA and BNST. Medial PBN damage, however, eliminates similar increased Fos expression on cortex and in both the CNA and the BNST. Lateral PBN destruction has little effect on Fos expression in any of these areas. The NAcS at most receives only a few direct projections from the PBN, and it is not known whether these axons convey gustatory activity (Refs. 18, 40, 71; Liang NC and Norgren R, unpublished observations). Nevertheless, sham sucrose licking increases Fos expression more than a similar intake of water in the accumbens shell. This increase is blocked by mPBN lesions but not by thalamic damage. This provides functional evidence that gustatory afferent activity related to reward systems reaches the forebrain primarily over the PBN-limbic projections.

This assertion is supported by data from dialysis and lesion behavioral studies. Licking 0.3 M sucrose solution increases

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**Fig. 9. Digital photomicrographs of Fos immunohistochemical staining in coronal sections of limbic nuclei.**

A and B: nucleus accumbens from a Con and a mPBNx rat, respectively, both of which were sham licking sucrose. C and D: CNA from a Con and a TTAx rat, respectively, both of which were sham licking sucrose. E and F: BNST from Con rats sham licking water (+W) and sucrose (+S). ac, anterior commissure; BLA, basolateral amygdala; BMA, basomedial amygdala; GP, globus pallidus; ic, internal capsule; LV, lateral ventricle; NAcC, nucleus accumbens, core; NAcS, nucleus accumbens shell; opt, optic tract. Scale bar in E = 0.5 mm.
NAcS DA release during both real and sham feeding (2, 16, 17, 19). As with the Fos increase, the accumbens DA plume during sucrose sham feeding is not impaired by thalamic gustatory lesions but is substantially blunted by parabrachial damage (18). Similarly, PBN lesions prevent the acquisition of a conditioned taste aversion (CTA) and the expression of sodium appetite without necessarily changing gustatory detection thresholds (13, 52, 57, 63). As above, TTA lesions have little or no effect on CTA, Na appetite, or gustatory preference-aversion functions (56). Thus a variety of measures including the present Fos data implicate the PBN projections to the ventral forebrain in the affective responses arising from gustatory stimuli.

The current Fos data extend this converging evidence by demonstrating that the CNA and the BNST, two areas that receive monosynaptic projections from PBN taste neurons (38), are candidates for processing central gustatory activity that reaches the nucleus accumbens (NAc). Compared with water, sham sucrose intake increases Fos label in all three of these nuclei. Damage to the mPBN eliminates the differential effect of sucrose licking in all three areas. As mentioned above, the NAc receives few if any taste axons from the PBN but substantial projections from the CNA and BNST (7, 40). This coincidence invites speculation that the CNA and BNST are part of the system through which gustatory afferent activity becomes rewarding. Although lesions of these structures have at best modest influence on taste-guided behaviors (30, 53, 64), the effect of CNA or BNST lesions on the response of NAc neurons to sucrose intake has yet to be tested.

Despite this converging evidence, the LH and the VTA, two areas long associated with ingestive behavior and reward and directly connected to the NAc, exhibited no increase in Fos during sham sucrose intake (24, 61). It is commonplace of the Fos technique that whereas an increase in label reflects a sustained increase in neural activity, the lack of a difference does not imply a steady state. Even so, given the clear Fos increases in limbic nuclei that are both anatomically and functionally related to LH and VTA, the lack of effect in these areas begs comment. One explanation could be the small sample size in the lesioned groups (n = 3 each), which might lead to false negatives. Without additional data, this possibility cannot be excluded, but an examination of Fig. 8 makes it appear less likely. In the VTA, the averages and variability of Fos profiles were essentially identical across conditions (sucrose vs. water) and lesion groups. In the LH, the three groups with central lesions had more Fos profiles when rats licked sucrose compared with water. Although not statistically significant, the differences are opposite to the predicted change. Because the lesions were in or adjacent to gustatory relays, damage to those areas should reduce rather than increase taste-driven neural activity. Increasing the sample size might reduce these differences, but it seems unlikely that it would reverse them.

The lack of a Fos increase in the VTA is notable not so much because the label increases in one of its target nuclei, the NAcS, but because of the parallel release of DA there during sucrose intake (16, 19). Two scenarios could account for this apparent disjuncture. First, during sucrose licking, VTA activity might change its pattern rather than the average rate. In fact, short bursts of activity in midbrain DA neurons have been linked to reinforcing events (51, 59, 60). Second, the modulation of DA release could occur within the NAc itself. Neurons in the NAc shell do increase the expression of Fos during sham intake of sucrose, but our data do not permit us to identify which cell type is involved.

The lack of a Fos response in the lateral hypothalamus is more complicated because of the reciprocal connections between LH and the PBN, NAc, and VTA as well as the amygdala and BNST (4, 28, 29). The patterning argument can be applied to the LH as well as the VTA (or any other area in which Fos fails to increase, for that matter). Nevertheless, at least for feeding behavior, the LH is viewed as an output structure, i.e., its projections to the hindbrain modulate the pattern generators that produce ingestive behavior (32, 55). If this is the case, then the lack of a Fos increase during sham sucrose intake becomes more plausible. Ingestion and rejection involve the same muscles operating in different sequences. Thus the same motoneurons fire during both behaviors, but in different patterns (65, 66). In fact, the motoneurons involved, largely in the trigeminal, facial, and hypoglossal nuclei, have interneuron pools that are indistinguishable from one another (10, 11, 12). Regardless, in the current experiment, the behavior did not switch from ingestion to rejection; sucrose simply elicited more intake than water. As mentioned above, increasing fluid intake alone was not sufficient to increase Fos. Therefore, the lack of a Fos effect in the LH during sucrose sham licking may be unexpected, but it is not outlandish.

In summary, these data demonstrate that gustatory neural activity produced during sham sucrose ingestion influences cells in the nucleus accumbens shell via the parabrachial ventral pathway rather than the thalamocortical loop. Beyond supporting this dichotomy, however, the same data cannot specify the neural systems needed to support the increased Fos in the accumbens. This will require both Fos and dialysis investigations in which PBN projection areas in the ventral forebrain are inactivated during sham sucrose intake. Nevertheless, the current experiment provides direct anatomical evidence of how the neural traces of an inherently preferred stimulus reach limbic systems associated with reward.

**Perspectives**

Reward is a famously shifty construct. It is apparent in daily living and essential to operant learning theory. When we attempt to follow reward into the brain, however, it vanishes in a welter of nuclei, neurotransmitters, and competing interpretations (40). The experiment summarized here attempts to pin down the neural traces relevant to a sensory event, the taste of sucrose, that people find rewarding and animals behave as if they do. Rodents appear to have a central gustatory system that differs fundamentally from Old World monkeys and probably humans (50). In monkeys, second-order gustatory neurons project monosynaptically from the nucleus of the solitary tract (NST) to the thalamus (3). Third-order thalamic cells then distribute to the primary taste cortex (48). In rodents, the NST-to-thalamus connection has an obligate synapse in the pontine PBN (41, 42). Parabrachial gustatory neurons then ascend to the thalamus as in primates, but they also project into the limbic system, most prominently into the lateral hypothalamus, amygdala, and bed nucleus of the stria terminalis (20, 37, 38). This bifurcation presents an opportunity, which was exploited in the current study, to determine how gustatory...
afferent activity reaches neural systems related to reward. Nevertheless, despite the differences in the central organization of this primary sensory system, monkeys also find sugars rewarding (49). Because they apparently lack the limbic taste projection, gustatory reward probably uses different central systems in Old World monkeys than in rodents. New World monkeys, carnivores, and ungulates may have still other variants (6, 21). Normally we conceive of model systems as ersatz versions of a human condition. In the present experiment, however, the differences between the rat and the (inferred) human central gustatory system permitted an approach to a question that transcends both the model and its object, the neural bases of reward. More prosaically, the differences remind us that model systems are only the flip side of comparative physiology.

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