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The Number and Location of Fos-like Immunoreactive Neurons in the Central Gustatory System Following Electrical Stimulation of the Parabrachial Nucleus in Conscious Rats

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Abstract

Electrical stimulation of the waist area (W) of the parabrachial nucleus (PBN) in conscious rats elicits stereotypical oromotor behaviors (Galvin et al. 2004). To identify neurons possibly involved in these behavioral responses, we used Fos immunohistochemistry to locate populations of neurons within central gustatory and oromotor centers activated by PBN stimulation. Dramatic increases in the numbers of Fos-like immunoreactive neurons were observed in the ipsilateral PBN, nucleus of the solitary tract (NST), and central amygdala. The increase in neurally-activated cells within the ventral subdivision (V) of the rostral NST is particularly noteworthy because of its projections to medullary oromotor centers. A modest increase in labeled neurons occurred bilaterally within the gustatory cortex. Although there were trends for an increase in Fos-labeled neurons in the gustatory thalamus and medullary reticular formation, most changes in labeled neurons in these areas were not statistically significant. Linear regression analysis revealed a relationship between the number of taste reactivity (TR) behaviors performed during PBN stimulation and the number of Fos-like immunoreactive neurons in the caudal PBN and V of the rostral NST. These data support a role for neurons in W of the PBN and the ventral rostral NST in the initiation of TR behaviors.

Key words: nucleus of the solitary tract, oromotor behavior, PBN, reticular formation, taste, taste reactivity

Introduction

Afferent fibers of the trigeminal, facial, glossopharyngeal, and vagus nerves carrying gustatory and oral somatosensory input terminate within the nucleus of the solitary tract (NST) in a topographic fashion, with gustatory fibers primarily ending in the rostral half of the nucleus (Hamilton and Norgren 1984). Based upon cytoarchitecture, the rostral NST in hamster and rat has been parceled into subdivisions that subserve different functions (Whitehead 1988; Halsell et al. 1996). For example, the rostral central subdivision (RC) receives the majority of the primary afferent input and contains most of the neurons that project to the parabrachial nucleus (PBN), a dorsal pontine structure that surrounds the brachium conjunctivum, whereas neurons in the ventral subdivision (V) project to the subjacent medullary reticular formation (RF; Fulwiler and Saper 1984; Whitehead 1990; Halsell et al. 1996; Gill et al. 1999; Travers and Hu 2000). The RF contains premotor circuits responsible for behavioral responses to orosensation (Travers et al. 1997, 2000; Chen et al. 2001). Specifically, in the rat, the parvocellular reticular formation (PCRt) receives input from brainstem orosensory regions (Travers and Norgren 1983; Halsell et al. 1996; Karimnamazi and Travers 1998) and activates trigeminal, facial, and hypoglossal motor systems either directly or via projections through the intermediate reticular formation (IRt, Ter Horst et al. 1991; Karimnamazi and Travers 1998; Cunningham and Sawchenko 2000).

The ascending orosensory projection from the NST terminates within specific subregions of the PBN (Norgren 1978; Travers 1988). The classic gustatory PBN region, the waist area (W), includes the central medial and ventral lateral subnuclei of the caudal PBN as well as the neuron bridges that span the brachium (Norgren and Leonard 1973; Norgren and Pfaffmann 1975; Halsell and Travers 1997). Although W is the termination site of the majority of ascending gustatory fibers, external PBN subnuclei also receive gustatory input (Herbert et al. 1990; Halsell and Travers 1997; Karimnamazi et al. 2002). Indicating a role for W in the initiation of oromotor behaviors, Galvin et al. (2004) showed that direct electrical stimulation of this subnucleus in conscious rats elicits ingestive taste reactivity (TR) behaviors

whereas stimulation of external PBN regions does not. The relatively heavy descending projection from W to V of the rostral NST and to PCRt, as compared with the projection originating in external PBN (Karimnamazi and Travers 1998), may provide an anatomical substrate for the differential behavioral effects of electrical stimulation of specific orosensory PBN subnuclei. On the other hand, the presence of ascending pathways from W to the thalamus, amygdala, hypothalamus, and insular cortex (Norgren 1976; Saper and Loewy 1980; Halsell 1992; Krukoff et al. 1993; Karimnamazi and Travers 1998; reviewed in Norgren 1995) suggests that TR responses elicited by stimulation of W may result from the activation of forebrain structures. Because most of the ascending orosensory pathways are reciprocal (Saper 1982; Shipley and Sanders 1982) and most of the forebrain areas that receive input from W have extensive projections to the brainstem (van der Kooy et al. 1984; Shammah-Lagnado et al. 1992; Whitehead et al. 2000; Hayama and Ogawa 2001), this explanation is reasonable. However, the maintenance of TR responses to gustatory stimuli in chronic supracollicular decerebrate rats (Grill and Norgren 1978b; Flynn and Grill 1988; Travers et al. 1999) strongly suggests that brainstem neural circuits are sufficient for TR responses to orosensory input.

The goals of the current study were to determine the location and number of neurons activated following electrical stimulation of W as well as the relationship of the active neurons to TR behaviors. Active neurons in brainstem and forebrain gustatory and oromotor centers were identified by their immunoreactivity for the Fos protein, the product of the immediate early gene *c-fos* (Morgan and Curran 1989; Sheng and Greenberg 1990). Recently, the detection of Fos-like immunoreactivity (FLI) in neurons has been used extensively within the central gustatory system (Yamamota et al. 1994; Harrer and Travers 1996; Streefland et al. 1996; DiNardo and Travers 1997; King et al. 1999; Travers et al. 1999; Travers and Hu 2000; Harrison 2001; Travers 2002; King et al. 2003). The current data suggest a significant role for neurons in the ventral rostral NST in generating oromotor responses following direct PBN stimulation. Preliminary data from this study have been presented in abstract form (King et al. 2003).

Materials and methods

Animals

Male Wistar rats weighing between 275 and 325 g were purchased from Hilltop Laboratories (Scottdale, PA) and housed individually in standard, hanging stainless steel cages. Rats were kept on a 12-h light–12-h dark cycle and given free access to water and Rodent LabDiet (PMI Nutrition International, Brentwood, MO). Data obtained from 13 rats are included in this report. All procedures were approved by the Institutional Animal Use and Care Committee and conform to guidelines of the National Institutes of Health.

Surgical implantation of electrodes

Stimulating electrodes, protective caps, and connecting wires were purchased from Plastics One (Roanoke, VA). The electrodes consisted of 2 stainless steel Formvar-insulated wires that were twisted around each other and protruded 7 mm below a plastic pedestal containing electrical mounts. The uninsulated tips of the wires were 150 μm apart.

Under sodium pentobarbital anesthesia (60 mg/kg intraperitoneally), rats were placed in a stereotaxic device with nontraumatic ear bars (Stoelting, Wood Dale, IL) with the skull held horizontal. The scalp was shaved, wiped with 70% ethanol, and a midline 2-cm incision made. A 1-mm burr hole was made in the skull over the right caudal PBN. An electrode was lowered vertically into the waist area of the right PBN using the stereotaxic coordinates of Paxinos and Watson (1998; 1.5 mm lateral to the midline, 9.3 mm caudal to bregma). The electrode was secured with dental acrylic and small screws embedded in the skull and covered with a cap to protect the electrical mounts. A topical antibiotic was applied and the skin sutured around and over the dental acrylic. Following surgery, rats were returned to their home cage, and examined and weighed each day to assess recovery.

Electrical stimulation of the PBN and behavioral assessment

Beginning on the fourth day after surgery, rats were habituated for 3 days to the behavioral arena. The behavioral arena was an opaque cylindrical plastic chamber that was 26 cm tall and 26 cm in diameter. The chamber was elevated by small metal pegs to 2.5 cm above a Plexiglas plate and placed on a glass table in the corner of a secluded room. A mirror below the table allowed videotaping of TR behaviors from below. Habituation entailed connecting the electrode to the electrical cable and placing the rat into the behavioral arena for 1 h without passing current.

On the following day (7 days after surgery), each rat was connected to the electrical cable and placed in the behavioral arena. After 1 h, the rat was videotaped with S-VHS equipment (Panasonic AW-E300 convertible camera and Sony SLV-R1000 video cassette recorder) for 2 min to assess the baseline level of oromotor behaviors. Then, using a Grass Instruments S48 stimulator and photoelectric stimulus isolation unit (W. Warick, RI), current was passed for a total of 8 min. The 8 min of stimulation occurred in four 2-min periods, each separated by 2 min of no stimulation. The current pulses (0.4-ms duration, 50 Hz, 100–400 μA) were identical to those shown in our previous study to elicit oromotor behaviors (Galvin et al. 2004). As a control, 4 rats experienced the same procedure, but no current was passed (unstimulated controls). Oromotor behaviors were videotaped during the stimulation period, including the intervening no-stimulation periods (a total of 14 min), and for an additional 2 min following the last stimulation.

Histology and Fos immunohistochemistry

After the stimulation protocol, the rats remained in the behavioral arena for 45 min before being anesthetized with an overdose of sodium pentobarbital (80 mg/kg intraperitoneally) and perfused intracardially with heparinized 0.15 M NaCl, followed by sodium phosphate–buffered 4% paraformaldehyde. The brains were removed and kept in the fixative overnight at 4 °C before being cut into 75-μm coronal sections using a vibratome. Every other section was processed for Fos immunoreactivity. These sections first were treated with 1% sodium borohydride in potassium phosphate–buffered saline (KPBS) for 20 min. After several rinses in KPBS, the sections were incubated with Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:10 000 in KPBS containing 0.4% Triton X-100 for 72 h at 4 °C. The sections then were rinsed with KPBS and incubated in biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) diluted at 1:600 in KPBS for 4 h at room temperature. After rinsing in KPBS, sections were placed in the reagents of a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) overnight at 4 °C. Then, the sections were rinsed in KPBS and placed in sodium phosphate buffer containing 0.03% diaminobenzidine, 0.008% nickel ammonium sulfate, 0.008% cobalt chloride, and 0.0075% hydrogen peroxide for 10–15 min at room temperature. Finally, the sections were rinsed and mounted on gelatin- and chrome alum–coated glass slides, dehydrated, and coverslipped using Permount (Fisher Scientific, Fair Lawn, NJ). The remaining alternate sections were mounted on coated slides, stained with 0.1% thionin, and coverslipped.

Microscopic analysis

The number and specific location of neurons that expressed FLI were determined bilaterally in 6 brain regions that receive orosensory input and/or are related to the control of oromotor behaviors: the PBN, the NST, the medullary RF, the gustatory thalamus (GT), the gustatory cortex (GC), and the central nucleus of the amygdala (ceA). As a control for the Fos labeling procedure, the CA3 region of the hippocampus also was examined for FLI. These regions and their associated subregions were identified in the Nissl-stained tissue viewed on a Zeiss Axioskop light microscope equipped with a video camera. The corresponding Fos-stained sections were then video captured onto a computer, the regions and subregions of interest outlined, and the number of neurons expressing FLI counted manually. The labeled neuron counts were performed by an experimenter who was unaware of the behavioral response outcomes.

The PBN was split into 4 subregions based on the descriptions of Fulwiler and Saper (1984): the waist region (including central medial and ventral lateral subnuclei), the external subregion (including external medial and external lateral subnuclei), the lateral PBN (the rest of the nucleus dorsal to the brachium conjunctivum), and the medial PBN (the rest of the nucleus ventral to the brachium conjunctivum). Of the 3 pontine sections analyzed, the waist region was present only in the caudal 2 sections whereas the external subregion was viewed only in the rostral 2 PBN sections. Four NST sections, spaced along the rostral–caudal plane of the nucleus, were analyzed. The first was in a nongustatory region at the level of obex. The second was just caudal to where the rostral NST begins (just caudal to where the nucleus moves lateral to the fourth ventricle). The third and fourth sections were at the level of entry of fibers of the IXth and VIIth cranial nerves into the solitary tract, respectively. Within the rostral 2 NST sections, the medial subdivision, RC, rostral lateral subdivision, and V were delineated as originally described in hamster by Whitehead (1988) and in rat by Halsell et al. (1996). Neurons expressing FLI within the RF were counted in the rostral 3 NST sections. The PCRt and IRt were identified using guidelines set by Travers et al. (1997). FLI neurons were counted in 3 sections of both GT and GC. GT was identified at the medial tip of the ventral posteromedial thalamic nucleus by noticing the relationship of the fasciculus retroflexus and the medial lemniscus (Norgren 1995; Paxinos and Watson 1998). The area of GC analyzed spanned from 0.0 to1.5 mm rostral to bregma and included the granular, dysgranular, and agranular insular cortex just dorsal to the piriform cortex, a region known to contain neurons that respond to gustatory input (Kosar et al. 1986; Cechetto and Saper 1987) and project to medullary taste centers (Whitehead et al. 2000; Hayama and Ogawa 2001). To assess a possible role of the pathway from the waist area of the PBN to the ventral forebrain in TR behaviors, FLI neurons were counted in the medial and lateral divisions of the central amygdala (ceA) in one section of the forebrain, just caudal to the optic chiasm (Whitehead et al. 2000).

Examination of oromotor behaviors

An experimenter, who was unaware of the tape sequence being analyzed, conducted a frame-by-frame analysis of videotape throughout the 18 min of the stimulation procedure (8 min of stimulation, 2 min before and after stimulation, and 2 min between each stimulation period). TR behaviors were scored using a previously described, standardized procedure (Grill and Norgren 1978a; Spector et al. 1988). Ingestive oromotor behaviors observed included mouth movements, tongue protrusions, and lip flares, whereas gapes, forelimb flails, chin rubs, and head shakes were categorized as aversive. Total TR scores reflect the sum of the occurrences of each individual oromotor behavior.

Data analyses

Rats were separated into 3 groups based upon whether current was applied and the number of TR behaviors performed by each rat during the entire stimulation procedure. Group 1

includes data from rats that received electrical stimulation and responded with 100 or more behaviors (n = 4). Group 2 includes data from rats that received electrical stimulation but responded with fewer than 100 behaviors (n = 5). And, Group 3 includes data from the unstimulated control rats (n = 4). Differences in the number of behaviors or neurons in a particular brain area expressing FLI among groups were determined using a single-factor analysis of variance (ANOVA). If the ANOVA revealed a significant group effect ($P < 0.05$), post hoc Fisher's least significant difference tests were performed to determine differences between Group 1 and the other 2 groups. A correlation between the number of ingestive TR behaviors performed by rats in Group 1 during stimulation and the number of FLI neurons in a particular brain region was determined by linear regression analysis.

Results

Stimulation of oromotor behaviors

The tips of the stimulating electrodes in rats in Group 1 were in the waist area (W), specifically the central medial subdivision (CM), of the right PBN (Figure 1), and stimulation at these sites elicited TR behaviors in conscious rats (Figure 2). Although the electrode tips in Group 2 rats were near W, they were either just ventral or medial to the PBN (Figure 1C and D), and stimulation at these sites caused fewer than 100 TR behaviors (Figure 2). These data confirm previously published findings indicating that stimulation of W (including CM and the ventral lateral PBN) initiates TR responses (Galvin et al. 2004). On average, the rats in Group 1 (stimulated and responded, n = 4) performed 12 and 7 times more TR behaviors than rats in Groups 2 (stimulated, did not

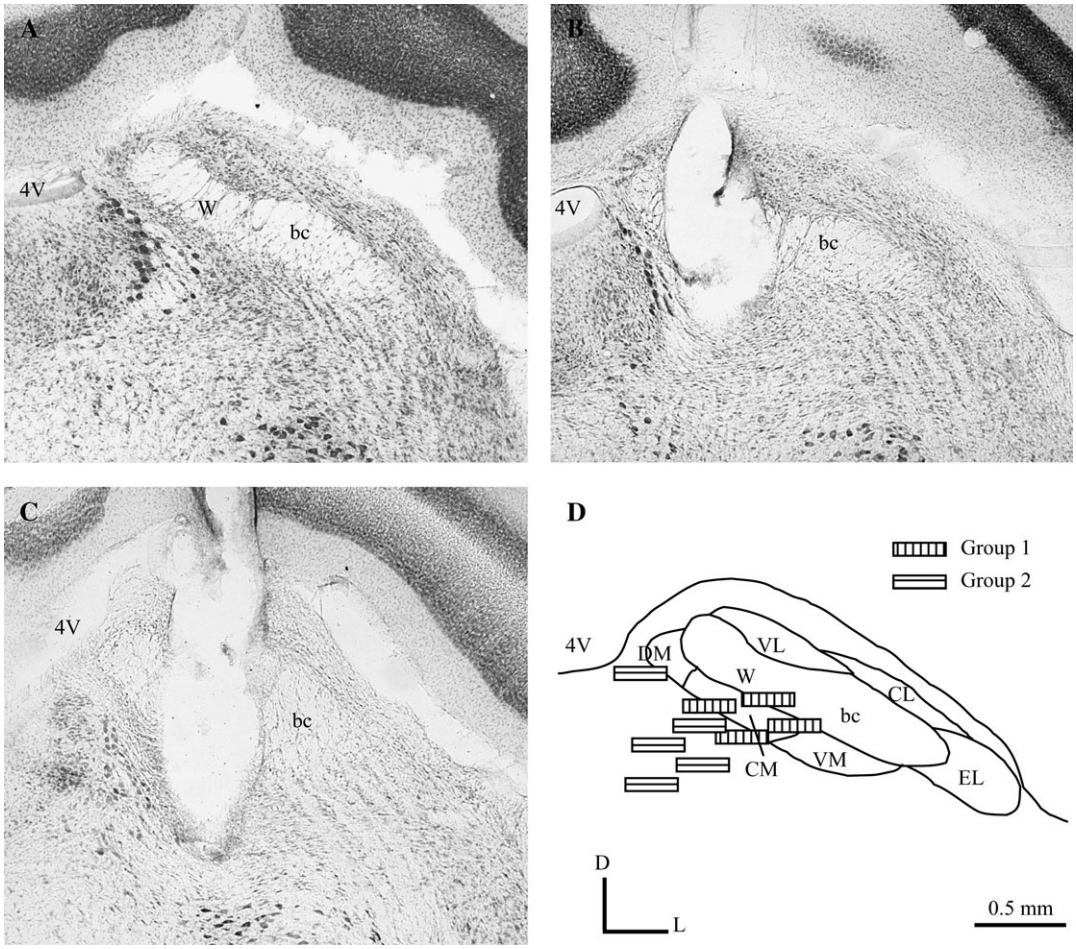

Figure 1 **(A)** Image of a Nissl-stained section of the right PBN showing the waist area, the target of the stimulating electrode. **(B)** Image of a Nissl-stained section showing a stimulation site from a rat in Group 1 (stimulated and responded). **(C)** Image of a Nissl-stained section showing a stimulation site from a rat in Group 2 (stimulated but did not respond). **(D)** Illustration of the location of the tip of the stimulating electrodes in all rats in Groups 1 and 2. The orientation of this sketch as well as the images in this figure is the same with top being dorsal and right lateral. Notice that although the stimulation sites intermingle, sites in Group 1 rats are within the CM subdivision of the PBN. The scale bar in D pertains to the images in A, B, and C as well. Abbreviations are 4V, fourth ventricle; bc, brachium conjunctivum; CL, central lateral PBN; DM, dorsal medial PBN; EL, external lateral PBN; VL, ventral lateral PBN; and VM, ventral medial PBN.

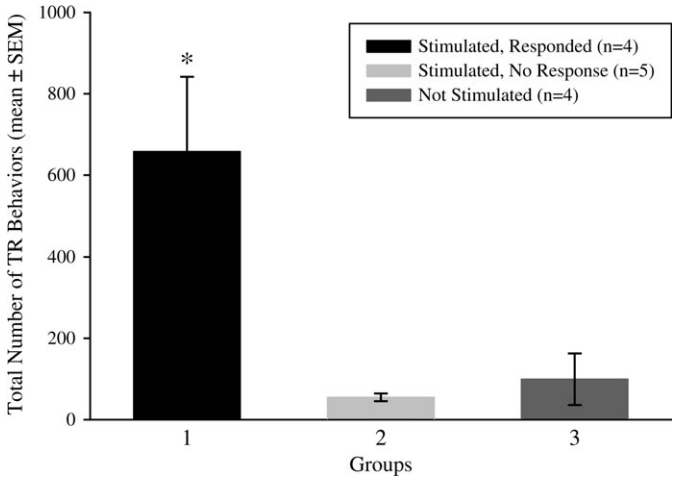

Figure 2 Mean (±SEM) total number of TR behaviors performed during the entire stimulation procedure (18 min) in the 3 groups of rats. Rats in Group 1 performed significantly more oromotor behaviors throughout the stimulation procedure than rats in Groups 2 and 3 (*$P < 0.05$ for ANOVA and post hoc tests).

respond, $n = 5$) and 3 (unstimulated controls, $n = 4$), respectively ($P < 0.05$, Figure 2).

The number and location of FLI neurons

Electrical stimulation of W more than doubled the number of neurons expressing FLI in the PBN in Group 1 as compared with the other groups ($F(2, 10) = 5.04$, $P = 0.03$, Figure 3). The largest increases in labeled neurons were in the right medial PBN ventral to the brachium conjunctivum ($F(2, 10) = 6.88$, $P = 0.01$) and in the external subnuclei ($F(2, 10) = 4.82$, $P = 0.03$). The significant increases in the number of Fos-labeled neurons were restricted to the right PBN, ipsilateral to the stimulation site (Figure 3B).

Because descending pathways from W terminate within the NST and RF, these regions of the medulla were examined for FLI. Stimulation of W caused a significant increase in the number of FLI neurons in the NST of rats in Group 1 as compared with rats in Groups 2 and 3 ($F(2, 10) = 5.73$, $P = 0.02$, Figure 4). A more than 3-fold increase in the number of labeled neurons in the right NST, ipsilateral to the PBN stimulation site, accounted for the overall FLI increase in the NST ($F(2, 10) = 7.90$, $P < 0.01$, Figure 4B). More specifically, the most dramatic augmentation in the number of FLI neurons in Group 1 occurred within the right V of the rostral NST ($F(2, 10) = 4.41$, $P = 0.04$, Figure 4C). There also was a significant, but smaller, increase in labeled neurons in the right RC in Group 1 ($F(2, 10) = 6.23$, $P = 0.02$, Figure 4C). An elevated number of FLI neurons was not confined to the gustatory portions of the NST as an increase in labeled neurons also was seen in the caudal NST at the level of obex ($F(2, 10) = 9.85$, $P < 0.01$). Interestingly, whereas the increase in FLI neurons in the rostral NST was ipsilateral to the PBN stimulation site, the increase in the caudal NST in Group 1

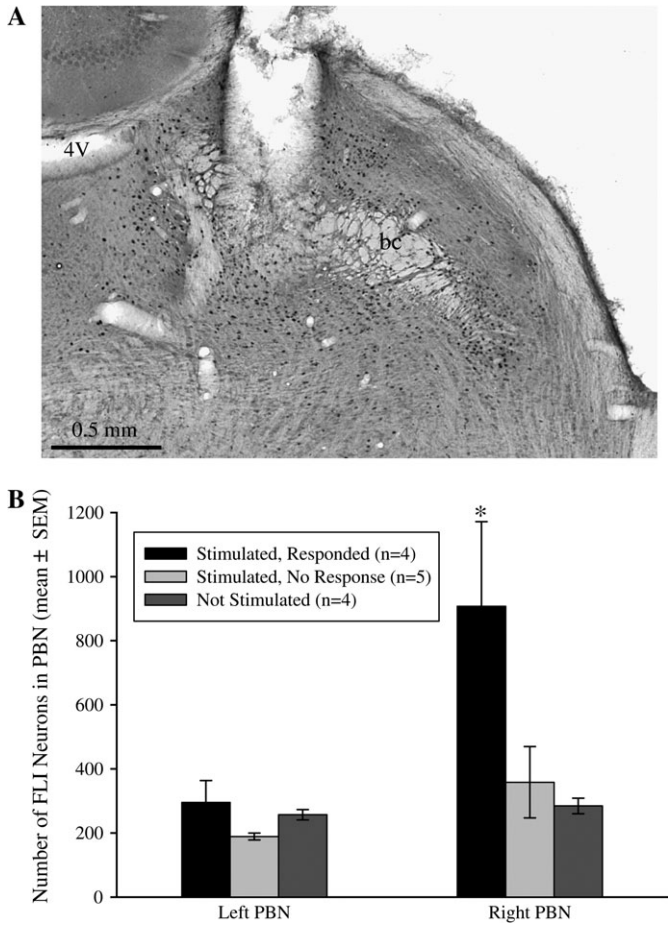

Figure 3 **(A)** Image showing FLI within the right PBN after stimulation of W in a Group 1 rat. This tissue section is just rostral to the stimulation site shown in Figure 1B. Notice labeled neurons surrounding the electrode damage, both dorsal and ventral to the brachium conjunctivum as well as in the external portions of the PBN (near the lateral edge of brachium conjunctivum [bc]). The orientation of the section and abbreviations are as in Figure 1. **(B)** Mean (±SEM) total number of FLI neurons in 3 sections of the PBN contralateral (Left PBN) and ipsilateral (Right PBN) to the stimulation site in each group of rats. Group 1 rats showed a significant increase in FLI in the right PBN as compared with rats in Groups 2 and 3 (*$P < 0.05$ for ANOVA and post hoc tests).

was bilateral (P's < 0.01). Although in Group 1 rats there were trends for more neurons expressing FLI within the RF overall ($F(2, 10) = 1.76$, $P = 0.22$), in the right RF as a whole ($F(2, 10) = 3.18$, $P = 0.09$), and in the PCRt ($F(2, 10) = 2.58$, $P = 0.13$) and IRt ($F(2, 10) = 3.61$, $P = 0.07$) ipsilateral to the stimulation site, none of these changes were statistically significant (Figure 5).

Ascending pathways from W travel to GC via a specific relay in GT, as well as directly to ceA; therefore, these areas also were examined for FLI elicited by PBN stimulation. Overall, the changes in the number of FLI neurons in GT and GC were smaller than those in the brainstem (1.5- to 1.8-fold increase as compared with 2.1–3.5, on average). Within GT ipsilateral to the PBN stimulation site, there was a modest increase in labeled neurons in Group 1

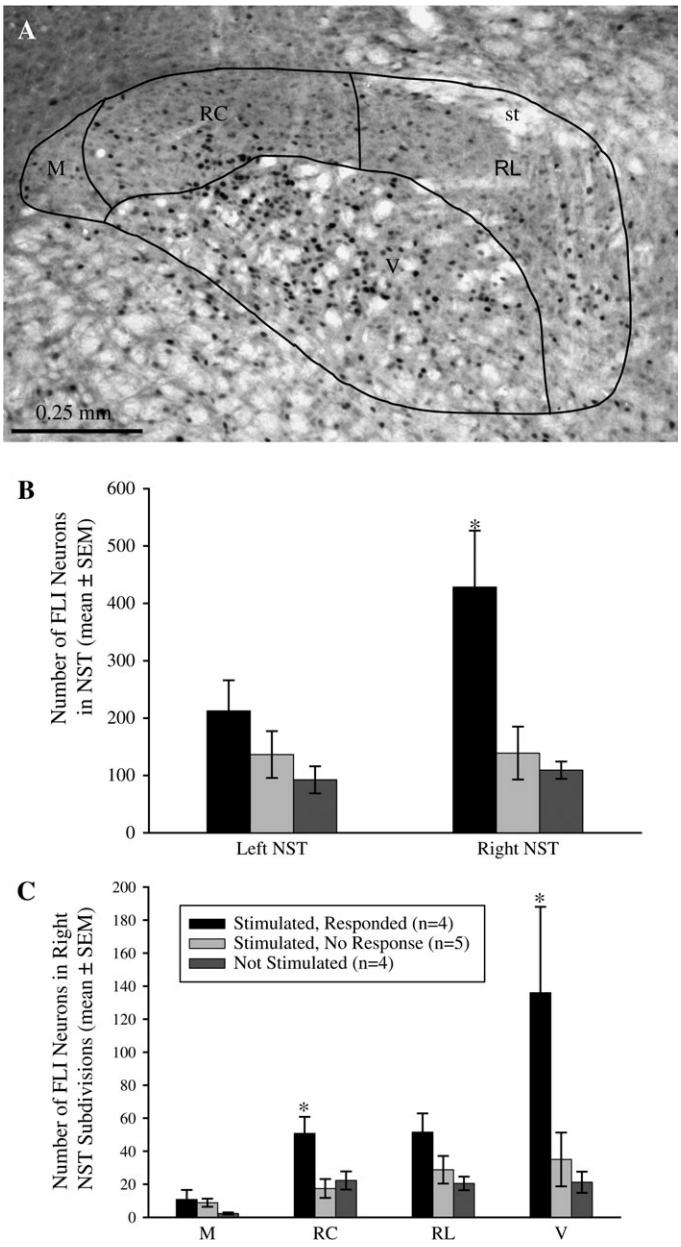

Figure 4 **(A)** Image showing FLI within the right NST after stimulation of W in a Group 1 rat. The subdivisions of the rostral NST are indicated. Notice abundant labeled neurons within V and subjacent RF. Left is medial and top is dorsal. Abbreviations are M, medial subdivision; RL, rostral lateral subdivision; and st, solitary tract. **(B and C)** Mean (±SEM) number of FLI neurons in 3 sections of the NST, not including the section at obex (B), and rostral NST subdivisions (C) in each group of rats. Most of the increase observed in the NST in Group 1 rats was due to an increase in FLI neurons in the right NST, specifically V and, to a lesser degree, RC (*P < 0.05 for ANOVA and post hoc tests comparing Group 1 with Groups 2 and 3).

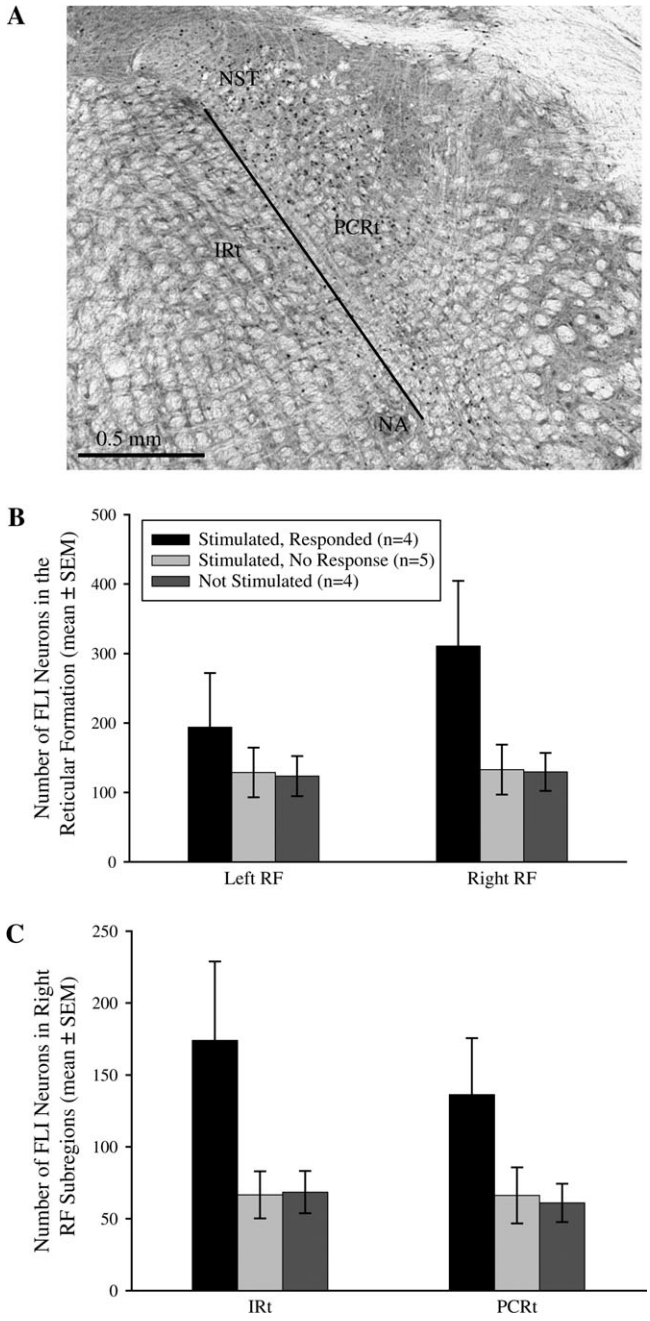

Figure 5 **(A)** Image showing FLI within the right medullary RF after stimulation of W in a Group 1 rat. The diagonal line indicates the boundary between the IRt and PCRt used to count FLI neurons. Left is medial and top is dorsal. Abbreviation: NA, nucleus ambiguus. **(B and C)** Mean (±SEM) number of FLI neurons in 3 sections of the RF, both contralateral (left RF) and ipsilateral (right RF) to the stimulation site (B) and in the right IRt and PCRt (C) in each group of rats. Although there was a trend for increased FLI neurons in Group 1 within each of the areas surveyed, the differences among groups were not statistically significant.

compared with the unstimulated controls (Group 3, $F(2, 10) = 4.57$, $P = 0.04$, Figure 6). The increase in FLI neurons was confined to the right side of the caudal-most GT section analyzed. Within GC, the increases in FLI neurons were more widespread, occurring bilaterally within 2 of the 3 GC sections observed (P's < 0.05, Figure 7). On the other hand, stimulation of W caused a dramatic increase in FLI neurons in ceA ($F(2, 10) = 9.12$, $P < 0.01$, Figure 8). The increases in FLI within the amygdala were particularly robust ipsilateral

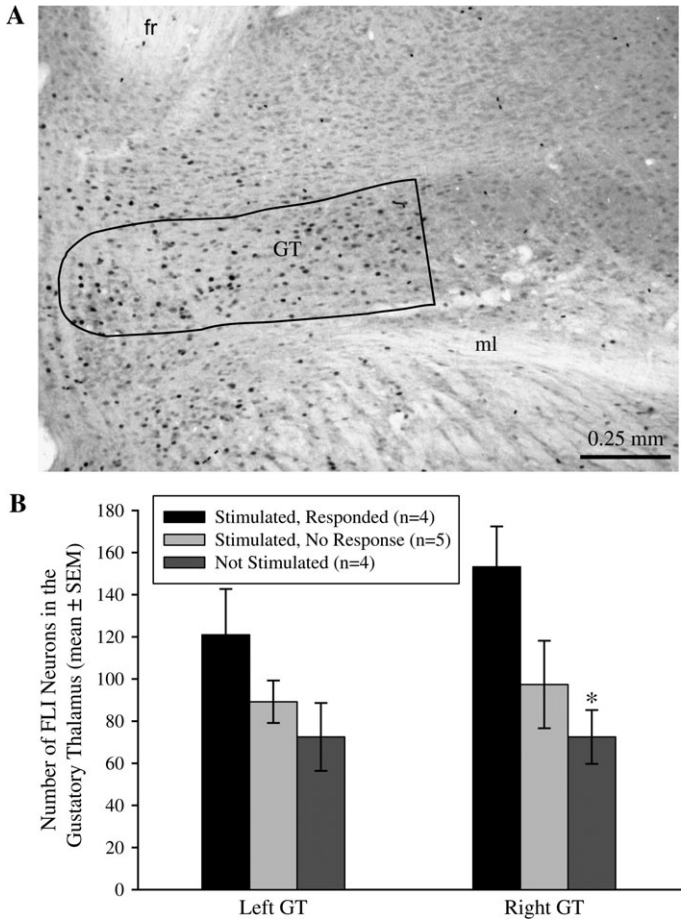

Figure 6 **(A)** Image showing FLI within the right GT after stimulation of W in a Group 1 rat. Left is medial and top is dorsal. Abbreviations are fr, fasciculus retroflexus, and ml, medial lemniscus. **(B)** Mean (±SEM) number of FLI neurons in 3 sections of the GT contralateral (left GT) and ipsilateral (right GT) to the PBN stimulation site. The only significant finding was a modest increase in labeled neurons in the right GT of rats in Group 1 compared with the unstimulated control rats (*$P < 0.05$ for ANOVA and post hoc test comparing Group 1 to Group 3).

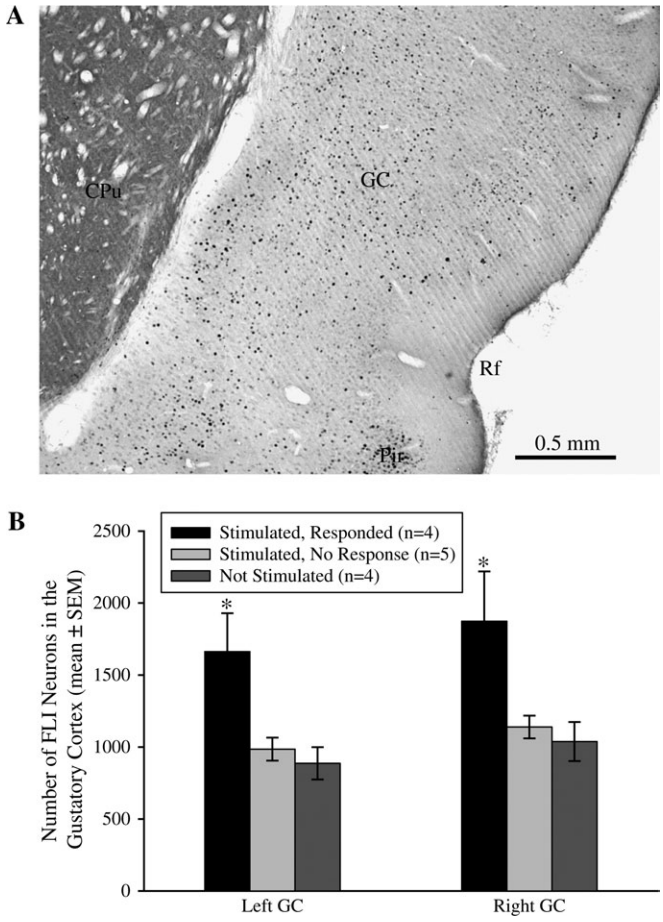

Figure 7 **(A)** Image showing FLI within the right GC after stimulation of W in a Group 1 rat. Left is medial and top is dorsal. Abbreviations are CPu, caudate putamen; Pir, piriform cortex; and Rf, rhinal fissure. Notice FLI neurons throughout GC. **(B)** Mean (±SEM) number of FLI neurons in 3 sections of the GC contralateral (left GC) and ipsilateral (right GC) to the PBN stimulation site (*$P < 0.05$ for ANOVA and post hoc tests comparing Group 1 to Groups 2 and 3). Within GC the dominance of an ipsilateral effect is lost as there were significant increases in FLI neurons on both the right and left sides.

to the stimulation site and included both the lateral and medial subdivisions of ceA.

Relationship between behaviors and FLI neurons

To determine if the number of neurons expressing FLI in a particular central gustatory or oromotor nucleus was related to behavior of rats in Group 1, linear regression analysis was performed on the number of TR behaviors during electrical stimulation of the PBN and the number of FLI neurons in each brain area analyzed (Figure 9, Tables 1 and 2). Within the brainstem, significant relationships were found between TR behaviors and FLI neurons in the caudal PBN and the NST ipsilateral to the stimulation site (Figure 9A, Table 1). In particular, the number of labeled neurons in the right waist area of the PBN (the stimulation site) and the right lateral PBN was strongly correlated to behavior (Table 1). Within the NST, the strongest relationship was found between behaviors and the number of FLI neurons on the right side of the rostral NST (Table 1). This correlation was mainly due to a strong relationship between the number of FLI neurons in V of the right rostral NST and TR behaviors (Table 1). Although PBN stimulation increased FLI in the caudal NST, the number of labeled neurons in the nongustatory NST was not correlated with TR behaviors. Whereas the number of behaviors tended to be higher in rats with more neurons expressing FLI in the RF, the neuron counts from the RF as a whole, as well as the PCRt and IRt individually, were not statistically related to behaviors (Table 1).

Fewer relationships were found between behaviors and FLI in the forebrain when compared with brainstem gustatory and oromotor centers (Figure 9B, Table 2). Specifically, the number of neurons expressing FLI bilaterally in the GT

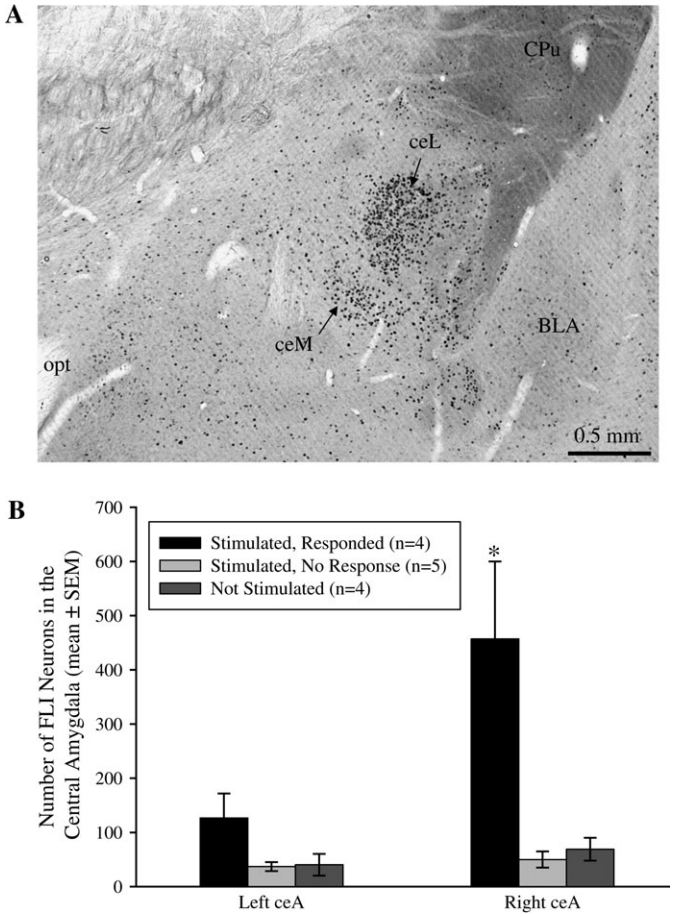

Figure 8 **(A)** Image showing FLI within the right ceA after stimulation of W in a Group 1 rat. Left is medial and top is dorsal. Abbreviations are BLA, basolateral amygdaloid nucleus; ceL, lateral division of the central amygdala; ceM, medial division of the central amygdala; CPu, caudate putamen; and opt, optic tract. Notice abundant FLI neurons within both the medial and lateral central amygdala. **(B)** Mean (±SEM) number of FLI neurons in 3 sections of the central amydgala contralateral (left ceA) and ipsilateral (right ceA) to the PBN stimulation site. The increase in the total number of FLI neurons within the central amygdala was mainly due to an increase ipsilateral to the stimulation site. Asterisk indicates that rats in Group 1 had significantly more FLI neurons than rats in the other groups ($P < 0.05$ for ANOVA and post hoc tests).

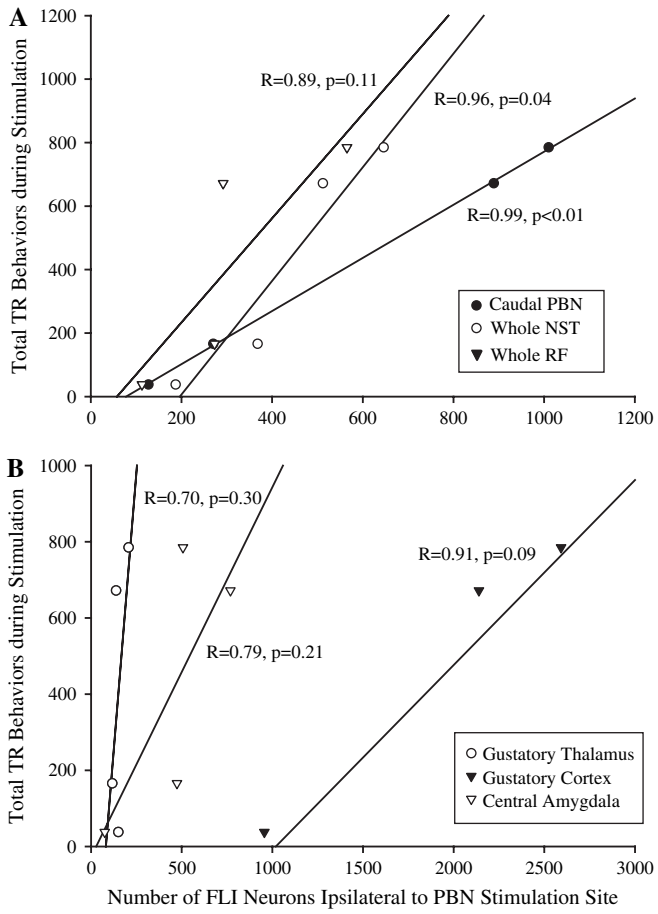

Figure 9 Total TR behaviors performed during the stimulation period versus the number of FLI neurons ipsilateral to the PBN stimulation site in brainstem **(A)** and forebrain **(B)** regions of the 4 rats in Group 1. The statistics indicated are the result of linear regression analyses. There was a strong relationship between the number of behaviors performed and FLI neurons within the right caudal PBN and NST but no correlation with labeled neurons in the right RF, GT, GC, or central amygdala.

($R = 0.55$, $P = 0.45$) and ceA ($R = 0.87$, $P = 0.13$), as well as just on the right side of these structures (Figure 9B, Table 2), was not related to TR responses, whereas correlations were found in only 2 subsections of GC. Although the overall number of labeled neurons in the right or left GC was not related to TR responses, the total number of FLI neurons in the middle cortical region analyzed (Table 2) as well as on the left side of the most rostral cortical section were correlated with behavior ($R = 0.98$, $P = 0.02$).

Discussion

Our previous study demonstrated that direct electrical stimulation of the waist region of the PBN (W) initiates ingestive oromotor behaviors in conscious rats (Galvin et al. 2004). The current study confirmed these previous behavioral findings and identified neurons in central gustatory and oromotor structures that are activated by PBN stimulation and therefore may be responsible for the behavioral responses. Although the number of active neurons, as assessed using Fos immunohistochemistry, within at least 1 subregion or section of all central structures analyzed was elevated in rats that received PBN stimulation and responded behaviorally, the most consistent and dramatic increases occurred within the PBN and the NST. Particularly interesting is the finding that the number of Fos-immunoreactive neurons in rostral V of the NST increased dramatically. This increase in FLI neurons in V undoubtedly reflects the strong projection from W to this rostral NST subdivision. Strongly suggesting that the activation of neurons in V is related to the behavioral response to PBN stimulation, the number of FLI neurons in V was

Table 1 Statistics from linear regression analysis of brainstem regions studied

Brain region	Subregion	*R*	*P*
PBN	Whole	0.89	0.11
	Rostral (1 section)	0.42	0.58
	Caudal (2 sections)	0.99	0.01*
	Waist	0.96	0.04*
	External	0.58	0.42
	Medial	0.64	0.36
	Lateral	0.96	0.04*
NST	Whole (-obex)	0.96	0.04*
	Rostral (2 sections)	0.98	0.02*
	Caudal (obex)	0.10	0.90
	Medial	0.84	0.16
	Rostral central	0.64	0.36
	Rostral lateral	0.70	0.30
	Ventral	0.99	0.01*
RF	Whole	0.89	0.11
	PCRt	0.92	0.08
	IRt	0.86	0.14

FLI neurons analyzed were on the right side of the brain, ipsilateral to PBN stimulation.
*$P < 0.05$

Table 2 Statistics from linear regression analysis of forebrain regions studies

Brain region	Subregion	*R*	*P*
Gustatory thalamus	Whole	0.70	0.30
	Rostral section	0.63	0.37
	Middle section	0.33	0.67
	Caudal section	0.40	0.60
Gustatory cortex	Whole	0.92	0.09
	Rostral section	0.94	0.06
	Middle section	0.95	0.05*
	Caudal section	0.79	0.21
Central amygdala	Whole	0.79	0.21
	Lateral	0.75	0.25
	Medial	0.78	0.22

FLI neurons analyzed were on the right side of the brain, ipsilateral to PBN stimulation.
*$P < 0.05$

significantly correlated with the number of ingestive TR behaviors performed. Therefore, the current findings support the supposition, based upon anatomical data (Halsell et al. 1996; Travers et al. 1997), that neurons in V of the rostral NST are premotor neurons that receive descending input from the gustatory PBN and activate brainstem motor circuits leading to behavior.

Technical considerations

Despite the limitations of using Fos immunoreactivity to map active central neurons, including the selective expression of the protein by a subset of active neurons (Dragunow and Faull 1989) and possible weak labeling of neurons near the end of multisynaptic pathways, this technique has been used successfully to map central neurons activated by sensory stimulation including gustatory input (Yamamoto et al. 1994; Harrer and Travers 1996; Streefland et al. 1996; DiNardo and Travers 1997; Travers et al. 1999; King et al. 2003). Furthermore, FLI has been used to map neurons activated by electrical stimulation of the hypothalamic paraventricular nucleus (Krukoff et al. 1994), central amygdala (Petrov et al. 1996), and lateral (cardiovascular) PBN (Krukoff et al. 1992) in anesthetized rats. By conducting our study in conscious rats with implanted electrodes, we avoided the influence of anesthesia on central neural activity and were able to observe behavioral consequences of CNS stimulation.

In the current study, FLI neurons were counted manually when the label was clearly discernable from the background tissue, but labeled neurons were not classified based on label intensity. Previous studies that separated neurons expressing FLI based on label intensity found no difference in their distribution following gustatory stimulation (DiNardo and Travers 1997; King et al. 1999). Attesting to the consistency of the Fos labeling and counting procedures employed in the current study, the average number of neurons expressing FLI in the CA3 region of the dorsal hippocampus (a region not considered to be influenced by PBN stimulation) was within a narrow range (23.5–26.9) among the 3 experimental groups of rats.

The electrode configuration and stimulation parameters used in the current investigation were selected to stimulate discrete brain regions (Valenstein and Beer 1961; Stark et al. 1962; Ranck 1975) and to elicit TR behaviors (Galvin et al. 2004). The ability to activate discrete PBN regions using these parameters was demonstrated by the results of our previous study (Galvin et al. 2004) as well as in the current study by the fact that rats in Group 2 were stimulated but did not respond behaviorally even though the electrode was placed just medial or ventral to W (Figure 1D). Also, in the current study, as in the preceding one, very few aversive TR behaviors were initiated by PBN stimulation. The specific location of the stimulation site and the pattern of electrical stimulation may have limited the possible behavioral outcomes. Indeed, it has been shown that the pattern of electrical activity in the NST and PBN is different for different tastants and may influence the behavioral responses elicited

(Perrotto and Scott 1976; Di Lorenzo and Schwartzbaum 1982; Di Lorenzo and Hecht 1993; Nishijo and Norgren 1997; Di Lorenzo et al. 2003). Nevertheless, the stimulation parameters used in the current investigation reliably elicited TR responses and FLI. Because central electrical stimulation activates fibers of passage as well as neuronal somata at the stimulation site, from the current study we cannot definitively conclude that the effects of PBN stimulation on behaviors and FLI were due to the activation of neurons in W. However, based on our previous findings that injection of the excitatory neurotransmitter glutamate into W initiates oromotor behaviors and that electrical stimulation ventral, medial, and lateral to W fails to cause a behavioral response (Galvin et al. 2004), it is clear that the activation of neurons in W is sufficient to elicit TR responses in conscious rats. Therefore, it is very likely that the behavioral and FLI responses to electrical stimulation in the current study were due to the activation of neurons in W.

A previous study found a strong correlation between the numbers of FLI neurons in regions of the RF involved in oromotor control and aversive TR responses (DiNardo and Travers 1997). Therefore, we hypothesized that similar relationships might exist between the brain regions and the TR behaviors we examined in the current study. We chose to restrict our analyses to the 4 rats in Group 1 principally because we felt that including rats that received misplaced stimulation (Group 2) and no stimulation (Group 3) would artificially improve the outcome of the analysis. That is, in these animals, there was little FLI and few behaviors. Indeed, when all 13 rats used in our study were included in the analysis, TR behaviors were significantly correlated with FLI in nearly every brain region. Restricting the analysis to the 4 animals that reliably showed FLI neurons and performed TR behaviors may have masked potentially significant relationships; but importantly, it served to identify more stringently the areas with the strongest relationship between neural activity and behavior. Finally, it is entirely possible that the degree of FLI expression (intensity rather than number) in a subset of neurons is the more critical determinant of behavioral output. Nonetheless, our data do show that the number of active neurons in particular brain regions is related to the number of ingestive TR behaviors performed.

Finally, although we assume that Fos expression is due to electrical stimulation of the PBN, it is possible that some FLI is induced by sensory feedback following TR behaviors elicited by central stimulation. The occurrence of the majority of FLI ipsilateral to the PBN stimulation site strongly suggests that most neurons are labeled because of electrical stimulation. In addition, the fact that one of the unstimulated control rats performed over 200 behaviors during the experiment but had very few FLI neurons in gustatory and oromotor centers argues against a significant influence of sensorimotor feedback in the induction of FLI in the current study.

Central neural substrate of oromotor behaviors

As expected, direct electrical stimulation of the waist area of the PBN elicited TR behaviors and increased the number of neurons expressing FLI within this nucleus ipsilateral to the stimulation site. The relationship between labeled neurons in the PBN and TR behaviors (Figure 9, Table 1) suggests that activity in W strongly influences behavioral output. The increase in FLI neurons in the external PBN is interesting because that region also receives gustatory input (Halsell and Travers 1997; Karimnamazi et al. 2002). Based on the ability to anatomically and behaviorally discern stimulation sites in these 2 PBN subdivisions in our previous study (Galvin et al. 2004), it is assumed that electrical stimulation did not spread directly from the electrode in W to the external PBN. Instead, it is more likely that in the external PBN, neuronal expression of FLI is the result of intranuclear projections or multisynaptic pathways originating in W and relayed through forebrain structures (Travers et al. 1999). Regardless of their mechanism of activation, the number of FLI neurons in the external PBN was not correlated with the number of TR behaviors performed, suggesting an alternative role for this PBN subregion.

The current findings confirm previous anatomical data demonstrating a predominantly ipsilateral projection from W to the NST (Herbert et al. 1990; Krukoff et al. 1992). This descending pathway preferentially terminates in V of the rostral NST (Karimnamzai and Travers 1998), as shown by the large increase in the number of FLI neurons in this subnucleus after PBN stimulation. As the main source of the projection from the NST to motor circuits in the medullary RF (Beckman and Whitehead 1991; Halsell et al. 1996), the activation of neurons in V would be expected to influence oromotor behaviors. In fact, such a role is suggested by the strong linear relationship found between the number of FLI neurons in V and the number of ingestive TR behaviors performed (Table 1). The current data also indicate that neurons in RC receive descending input from W. Because the number of active neurons in RC was not correlated with behaviors, it is likely that instead of altering motor output directly the descending projection from the PBN to this subdivision modulates activity in the ascending gustatory pathway. The current data also suggest that the projection from W to the NST in not confined to the gustatory portions of the NST but instead also influences the caudal-most regions of this nucleus. Because the linear regression analysis implicates a role for specific populations of neurons in the rostral, but not caudal, NST in TR behaviors, the descending projection from the PBN to the caudal NST may regulate visceral functions related to TR behaviors but not the behaviors themselves.

Although electrical stimulation of the PBN caused dramatic increases in FLI in the PBN and NST, only modest increases were noticed in the medullary RF. In fact, the only significant increase in FLI neurons was found in 1 section of

the right side of RF at the level of entry of fibers of the IXth nerve into the solitary tract. The lack of an increase in FLI neurons in RF was not expected because premotor neurons responsible for licking and other oromotor behaviors are located in this region (Travers et al. 1997; Chen et al. 2001). It was particularly surprising not to see an increase in active neurons in the PCRt, the site of termination of direct projections from the PBN (Herbert et al. 1990; Karimnamazi and Travers 1998) and V of the rostral NST (Norgren 1978; Travers 1988; Beckman and Whitehead 1991; Shammah-Lagnado et al. 1992; Halsell et al. 1996). It is possible that PBN stimulation was not strong enough or in the correct pattern to elicit Fos expression in RF neurons. It is also possible that there would have been more FLI in RF if aversive behaviors were elicited by PBN stimulation. This latter possibility is supported by data demonstrating a correlation between the number of RF neurons expressing Fos and the number of gapes performed to intraoral quinine, but not TR responses to sucrose (DiNardo and Travers 1997).

Despite a direct, primarily ipsilateral, projection from W to the GT (Fulwiler and Saper 1984; Karimnamazi and Travers 1998), only a small (60%) increase in the number of FLI neurons was found on the right side of one tissue section of GT following PBN stimulation. In addition, there was no relationship between the number of labeled thalamic neurons and TR behaviors performed. These data are consistent with a role for the GT in the preparatory, but not consummatory, phase of taste-guided behaviors (Reilly 1998). On the contrary, there were increases in the number of FLI neurons throughout the GC, but due to a high number of labeled neurons in unstimulated rats, the percentage increases in labeled cortical neurons following PBN stimulation were not as large as in the brainstem. The presence of a direct connection from W to GC that bypasses the thalamus (Lasiter et al. 1982; Saper 1982; Shipley and Sanders 1982) may account for the increase in cortical FLI neurons without a corresponding change in labeled GT neurons. The increases in FLI neurons in GC were not well correlated with behavior. Because the activation of ascending pathways primarily subserves higher cognitive functions associated with gustation, this finding is not surprising.

The waist area of the PBN gives rise to 2 ascending pathways: one that relays through GT to the insular cortex and another that travels directly to the ventral forebrain (Saper and Loewy 1980; Lasiter et al. 1982; Saper 1982; Krukoff et al. 1993; reviewed in Norgren 1995). A possible role for the ventral forebrain projection in TR behaviors elicited by PBN stimulation was assessed by examining FLI within the ceA, one of the main targets of this pathway and an area with significant descending projections (van der Kooy et al. 1984; Petrov et al. 1996; Whitehead et al. 2000). Confirming the presence of a strong projection from W to the central amydgala, electrical stimulation caused a large increase in the number of FLI neurons in both the lateral and medial subdivisions of this nucleus, particularly ipsilateral to the stimulation site. However, the lack of a relationship between FLI in the central amygdala and TR behaviors suggests that the activated amygdalar neurons may not play a critical role in the initiation of oromotor behaviors. Previous electrophysiology findings suggest that descending projections from the central amygdala may play a modulatory role in the processing of gustatory input within the brainstem (Lundy and Norgren 2001; Li et al. 2002, 2005).

Conclusions

Activation of the classic pontine gustatory center (waist area of the PBN) elicited TR behaviors in conscious rats. The number of behaviors performed was related to the number of active neurons, as detected using Fos immunohistochemistry, in the PBN as well as in V of the rostral NST, which contains neurons that project to medullary premotor centers. These findings suggest that TR behaviors following direct electrical stimulation of the PBN are due to the activation of a descending pathway to the medulla and imply that such a pathway may regulate oromotor responses elicited by infusion of solutions into the oral cavity. The latter hypothesis is being addressed by pharmacological blockade of activity within the PBN during intraoral infusion of taste solutions.

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