

Research report

Subdiaphragmatic vagotomy does not attenuate c-Fos induction in the nucleus of the solitary tract after conditioned taste aversion expression

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Abstract

After acquisition of a conditioned taste aversion (CTA) against sucrose, intraoral infusions of sucrose induce c-Fos-like immunoreactivity (c-FLI) in the medial intermediate nucleus of the solitary tract (iNTS) of the rat. In order to determine if c-FLI expression in the iNTS depends on subdiaphragmatic vagal afferent input to the NTS secondary to gastrointestinal symptoms during CTA expression (e.g. diarrhea), we quantified the induction of c-FLI in the iNTS by sucrose infusions after total subdiaphragmatic vagotomy in rats with a previously acquired CTA against sucrose. Rats were conditioned against intraoral infusions of sucrose by pairing sucrose infusions with toxic LiCl injections. After CTA acquisition, rats underwent bilateral subdiaphragmatic vagotomy or were sham-vagotomized. One week after surgery, rats received an intraoral infusion of sucrose. One hour after the test infusion, rats were perfused and processed for c-FLI. Vagotomy had no apparent effect on the behavioral expression of the previously acquired CTA, because both vagotomized and sham-vagotomized rats rejected all of the test intraoral infusion of sucrose. There was also no significant difference between vagotomized and sham-vagotomized rats in the number of c-FLI-positive cells in the iNTS after CTA expression. We conclude that c-FLI induction correlated with CTA expression is not dependent on subdiaphragmatic vagal efferent output or afferent input.

Keywords: Gastrointestinal; Immediate early gene; Learning; Memory; Gene expression; Axotomy

1. Introduction

The expression of c-FLI in the iNTS is a neuronal correlate of behavioral CTA expression. Unpaired intraoral infusions of sucrose [14], saccharin [29], or quinine [12] induce c-FLI in the iNTS of rats that have acquired CTAs against sucrose, saccharin, or quinine by prior pairing of LiCl with intraoral infusions of the tastant. Intraoral infusions of sucrose [14] or quinine [12,30] do not induce c-FLI in the iNTS of unconditioned rats or in conditioned rats after extinction of the CTA. The c-FLI is specifically induced by the conditioned taste stimulus and not by unconditioned tastes [11], and can be induced by intraoral infusions of the taste stimulus up to six months after CTA acquisition [13].

The neural circuitry responsible for c-FLI expression in the iNTS is not known. Because c-FLI is a marker for transsynaptic activation of cells [18], the induction of c-FLI in the iNTS by a conditioned intraoral infusion of

sucrose must be due to activation of afferent terminals synapsing on cells of the iNTS that now produce detectable increases of c-FLI as a correlate of the expression of the CTA. This increased c-FLI response could reflect increased afferent activity or an increase of responsiveness of *c-fos* transcription in neurons of the iNTS.

The NTS has widespread, reciprocal connections with medullary, pontine, and forebrain nuclei, and receives afferent gustatory, visceral, and chemoreceptive information from a variety of sensory pathways [1]. Thus there are many candidate pathways upon which c-FLI induction may be dependent. The vagus nerve is one of these candidate pathways. The caudal half of the NTS is the first central relay for visceral information relayed by afferent fibers of the abdominal vagus nerve [19].

The abdominal vagus has been shown to be necessary for acquisition of CTA when gastrointestinal toxins are employed as the unconditioned stimulus (e.g. [4]), but little work has been done on the role of the vagus during expression of a previously acquired CTA [16].

The subdiaphragmatic afferent vagus may detect the conditioned gastrointestinal responses accompanying CTA

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expression. The stimulation of the afferent vagus during CTA expression may lead to activation of first-order central neurons in the NTS. The c-FLI expression observed after CTA expression may therefore be secondary to vagal afferent input. In order to test this hypothesis, we quantified the induction of c-FLI in the iNTS by intraoral infusions of sucrose after total subdiaphragmatic vagotomy in rats that had acquired a CTA against sucrose. A preliminary report has appeared [9].

2. Materials and methods

2.1. Animals and intraoral catheterization

Adult male Sprague-Dawley rats (300 g) were individually housed under a 12:12 h light/dark cycle at 25°C. Food (powdered Purina rodent chow) and water were provided ad libitum except as noted below.

Anterior sublingual intraoral catheters were implanted under Metofane anesthesia as described previously [14]. Intraoral catheters were prepared from 10 cm of PE-50 polyethylene tubing; one end of the catheter was heat-flared to form a 2-mm diameter annular end. A small incision was made on the ventral midline between the mandibles, and a bent 23-gauge syringe needle pushed between the mandibles until the needle projected into the mouth midway between the root of the lower incisors and the base of the tongue. The unflared end of the catheter was affixed to the end of the syringe needle; the needle was retracted to pull the tubing along the needle tract and out the incision on the ventral submental surface until the flared end of the catheter rested on the floor of the mouth beneath the tongue.

An incision was then made from the caudal extent of the skull to midway between the scapulas on the dorsal surface of the rat's neck. A blunt wire probe was threaded between the skin and the musculature from the dorsal incision to the ventral submental incision. Then the end of the intraoral catheter was attached to the wire probe, which was pulled back with the intraoral catheter under the skin and externalized through the dorsal incision. The intraoral catheter was held in place by threading it through an outer sleeve of 0.040 silastic tubing attached to a 15-mm diameter Marlex mesh disk (Bard-Parker) sutured to the dorsal neck musculature. A 5-cm length of sleeve and catheter projected from the dorsal surface of the rat for attachment to an infusion catheter. The dorsal neck incision was closed with wound clips on either side of the catheter sleeve and the submental incision was sutured closed.

2.2. Conditioning

Twenty-one rats were implanted with intraoral catheters as described above and conditioned by pairing intraoral infusions of 5% sucrose (sucrose) with LiCl. Rats were deprived of food, but not water, for 17 h prior to intraoral infusions. Individual rats were weighed and placed in 26

cm wide by 17 cm deep by 30 cm tall test chambers (formed by subdividing a 40-gallon glass aquarium with Plexiglas walls). Syringe pumps (Harvard Apparatus) infused sucrose from 20-ml syringes at a rate of 1.1 ml/min for 6 min (setting 10) through 0.0040-gauge silastic catheters attached to the externalized end of the implanted intraoral catheters. Rats and any feces produced in the test chamber during the infusion were weighed immediately after the 6-min infusion; the weight gained during the infusion procedure was recorded as a measure of consumption of sucrose. The rats were returned to their home cages, and injected with LiCl (0.15 M, 12 ml/kg, i.p.) 30 min after the start of the intraoral infusion. Intraoral sucrose infusions were paired three times with LiCl injections at 48-h intervals. Although rats will form a CTA against intraoral infusions after a single pairing, we have found that a minimum of three pairings is required for rats to reject completely an intraoral infusion of 5% sucrose. All tests were conducted 2–6 h after lights on.

2.3. Subdiaphragmatic vagotomy

Seven to ten days after the last pairing of sucrose and LiCl, rats underwent either bilateral subdiaphragmatic vagotomy ($n = 11$) or sham vagotomy ($n = 10$). Bilateral subdiaphragmatic vagotomy was performed using the technique described previously [28]. Both major vagal trunks were cut between two 3-0 silk ligatures as high as possible on the esophagus below the diaphragm and above the hepatic, accessory celiac, and celiac branches. Sham vagotomy consisted of the same procedure without touching the esophagus or nerves. Rats were maintained on powdered Purina rodent chow after surgery. (One vagotomized rat with respiratory complications was treated postoperatively with atropine sulfate and maintained on a mixture of powdered chow and sweetened condensed milk and subsequently included in the non-infused, vagotomy control group; no difference was seen in his response.)

Vagotomy significantly affected body weight over the week after surgery. Vagotomized rats showed an average decrease in body weight of $19 \pm 4\%$ one week after surgery; the average body weight of sham-vagotomized rats decreased only $1 \pm 3\%$.

2.4. Test of CTA expression

Six to seven days after vagotomy or sham-vagotomy, all rats were 17-h food deprived. Some of the vagotomized rats ($n = 5$) and sham-vagotomized rats ($n = 5$) received a final intraoral infusion of 5% sucrose (6.6 ml over 6 min). No LiCl injection was administered. The rats were weighed immediately before and after the test infusion, and returned to their home cages at the end of the infusion. One hour after the start of the sucrose infusion, the rats were sacrificed and processed for c-FLI as described below.

The remaining vagotomized ($n = 4$) and sham-vagotomized ($n = 5$) rats were sacrificed and processed for c-FLI without receiving an intraoral infusion. These non-infused

control groups were included because preliminary studies revealed that substantial numbers of c-FLI-positive cells were present in the caudal NTS of rats 1 week after vagotomy (see below; [27]). Thus, non-infused groups were necessary to control for the surgically-induced c-FLI and to establish the independence of c-FLI induction after CTA expression.

2.5. Tissue collection and immunohistochemistry

Rats were overdosed with sodium pentobarbital and, when completely unresponsive, transcardially perfused first with 100 ml heparinized isotonic saline containing 0.5% NaNO₂, then with 400 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brains were dissected, blocked, post-fixed for 2 h, and transferred into 30% sucrose for cryoprotection. Forty- μ m coronal sections were cut on a freezing, sliding microtome through the rostral–caudal extent of the NTS. Thirty-six sections were cut from the caudal subpostremal NTS (bregma –14 mm) to the caudal extent of the rostral NTS (bregma –12.8 mm). All coordinates were based on Paxinos and Watson's atlas [20]. Every other section collected from the NTS was processed for c-FLI.

Free-floating tissue sections were washed twice for 15 min in 0.1 M sodium phosphate-buffered saline (PBS), then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS-BSA, sections were incubated overnight with a sheep anti-c-Fos peptide antibody (Cambridge Research Biochemicals) at a dilution of 1:3000. Sections were washed in PBS-BSA twice and incubated for 1 h with a biotinylated anti-sheep rabbit antibody (Vector Laboratories); bound secondary antibody was then amplified with the Vector Elite ABC kit. Antibody complexes were visualized by a 5-min 0.5% diaminobenzidine reaction.

Cells expressing positive, nuclear c-FLI were quantified in the medial intermediate NTS by hand with an MCID image analysis system (Image Research, Montreal) and the cell scoring macros of the NIH Image program (W. Rasband, NIH, Bethesda). Only dark, punctate nuclear staining was counted; diffusely stained cell bodies were not counted. Cells were counted medial to the solitary tract in four sections of the iNTS from each rat. The iNTS was defined as sections where the NTS abutted the IVth ventricle on two sides: the dorsal aspect and medial aspect (see Fig. 2 for examples). In order to restrict counting to the medial subnucleus of the iNTS, a dorsal–medial quadrant was delineated by drawing a vertical line from the dorsal–lateral corner of the iNTS (at the junction of the NTS, gracilis nucleus, and IVth ventricle) and drawing a horizontal line from the ventral–medial corner (at the junction of the NTS, dorsal motor nucleus, and IVth ventricle). These boundaries excluded most of the centralis subnucleus, located ventral and lateral to the medial subnucleus and immediately medial to the solitary tract.

Total bilateral cell counts for four sections of the medial iNTS of each rat were averaged, and the individual mean counts averaged across rats within experimental groups. All data were analyzed by analysis of variance and subsequent post-hoc analyses were performed using Fisher's post-hoc test.

2.6. Verification of vagotomy

After perfusion, rats were examined with the aid of a dissecting microscope (10 \times –40 \times). The thoracic vagal trunks were isolated by dissection and followed along the esophagus toward the stomach. The two silk ligatures on each vagal trunk were identified and the space between them examined and dissected to search for connections between the upper and lower nerve segment. Vagotomy was considered complete if no connecting fibers were identified. This was confirmed in all of the vagotomized rats.

3. Results

3.1. CTA expression

Vagotomy had no significant effect on the behavioral expression of the CTA acquired against sucrose before surgery as measured by intake during intraoral infusion of sucrose after surgery (see Fig. 1). One week after surgery, both vagotomized and sham-vagotomized groups did not gain much weight during an unpaired intraoral infusion of 5% sucrose (6.6 ml over 6 min); thus both groups rejected most of the sucrose infusion.

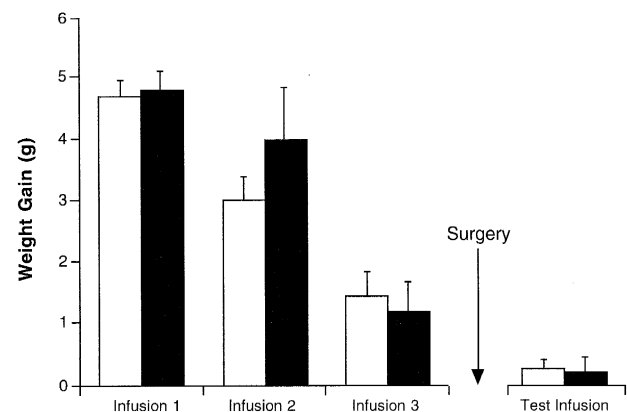
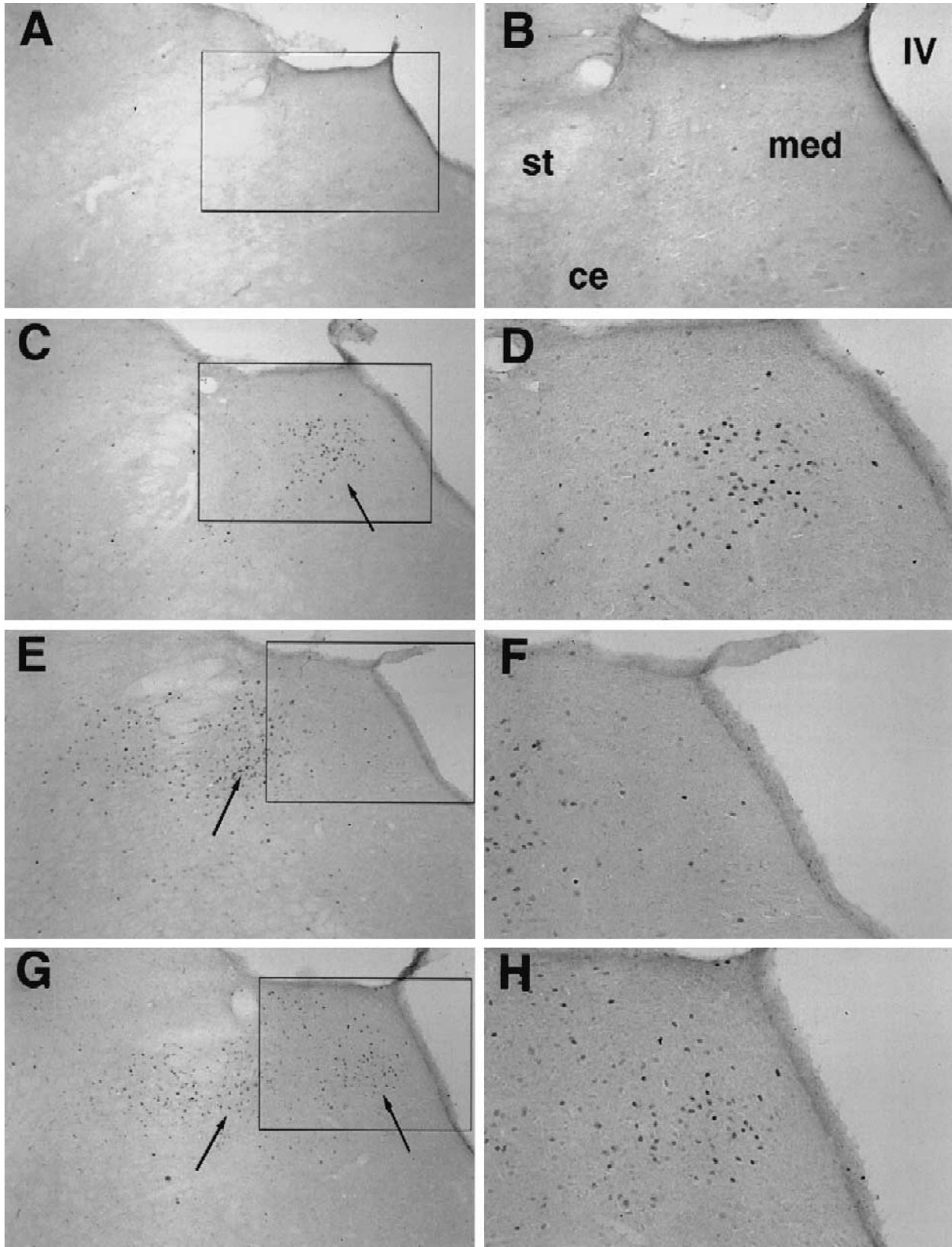


Fig. 1. Weight gained during intraoral infusions of 5% sucrose (6.6 ml over 6 min). The first three infusions were administered 30 min before injection of LiCl (0.15 M, 12 ml/kg i.p.) on alternate days prior to surgery; treatment was identical for both groups. Rats underwent vagotomy (black bars) or sham-vagotomy (white bars) 6 or 7 days after the third infusion. The final test infusion of 5% sucrose (6.6 ml) was administered 6–7 days after surgery. No significant difference in sucrose intake was seen between groups.

3.2. *c-FLI* in the *iNTS*

Significantly more *c-FLI*-positive cells were counted in the medial *iNTS* of rats receiving an intraoral infusion of

sucrose than in rats sacrificed without infusion in both vagotomized and sham-vagotomized groups (see Fig. 2 and Fig. 3). The *c-FLI*-positive cells specifically induced by the intraoral infusion of sucrose were clustered in a



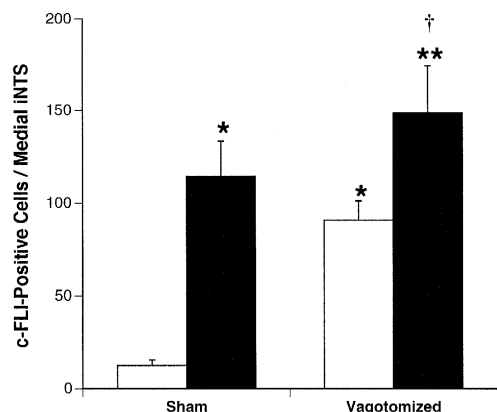


Fig. 3. Quantification of c-FLI induced in the medial iNTS by intraoral 5% sucrose infusion (6.6. ml over 6 min; black bars) or without infusion (white bars) in vagotomized or sham-vagotomized rats with CTAs acquired against sucrose. Sucrose infusions induced significantly more c-FLI-positive cells compared to non-infused rats in both vagotomized and sham-vagotomized groups; there was no significant difference in number of c-FLI-positive cells induced by sucrose infusion between the two groups, however. Both infused and non-infused vagotomized rats had significantly more c-FLI-positive cells than non-infused sham-vagotomized rats. * $P < 0.005$, ** $P < 0.0005$ compared to non-infused, sham-vagotomized rats. † $P < 0.05$ compared to non-infused, vagotomized rats.

circular distribution equidistant from the medial and dorsal aspects of the iNTS, and medial to the centralis subnucleus (which is ventral and medial to the solitary tract; [1]).

In addition to these c-FLI-positive cells that we have previously shown correlate with the expression of CTA, we also observed significantly more c-FLI-positive cells in other subregions of the NTS in both infused and non-infused vagotomized rats than in sham-vagotomized rats (see Fig. 2A, C, E, G). Intense c-FLI was observed in the centralis subnucleus of the iNTS, in the caudal, subpostremal NTS, along the area postrema-NTS border, and within the area postrema. At both the intermediate and caudal levels of the NTS, numerous c-FLI-positive cells were seen lateral to the solitary tract as well. In sham-vagotomized rats, almost no c-FLI-positive cells were observed in any of these areas in either the infused or non-infused groups. The majority of the c-FLI induced by vagotomy in the NTS was excluded from quantification by restricting cell-counts to the medial subnucleus of the iNTS.

We also observed c-FLI in more rostral regions of the brain of vagotomized rats (e.g. lateral parabrachial nucleus, central nucleus of the amygdala, and the hypothalamic

paraventricular and supraoptic nuclei), but analysis of these regions was beyond the scope of this study [27].

4. Discussion

Total subdiaphragmatic vagotomy did not attenuate the behavioral expression of a CTA against sucrose acquired prior to vagotomy as measured by intake of an intraoral infusion of sucrose after vagotomy. Thus, our results do not support the proposition that conditioned gastrointestinal responses elicited by the conditioned taste stimulus and mediated by abdominal vagal nerves are the proximal cause of behavioral responses during CTA expression under our conditions [3].

Although one previous study demonstrated that vagotomy increased intake of saccharin by water-deprived rats in single-bottle intake tests when a CTA against saccharin had been acquired before vagotomy [16], the attenuation of CTA expression by vagotomy was much larger for a CTA acquired with a vagally-mediated toxin (intragastric copper sulfate [4]) than for a CTA acquired with a postremal-mediated toxin (i.p. apomorphine [3,16]). Thus our failure to detect a difference in CTA expression after abdominal vagotomy may have been because we used LiCl, a blood-borne toxin that is dependent on the area postrema [2], but not the vagus [17], for acquisition of a CTA or on other procedural differences between the two experiments.

Vagotomy also did not attenuate induction of c-FLI in the iNTS after an intraoral infusion of sucrose in rats with a previously acquired CTA against sucrose. Therefore, the induction of c-FLI in the iNTS correlated with CTA expression is not dependent on subdiaphragmatic vagal efferent or afferent activity. This experiment does not rule out a role for other conditioned visceral and autonomic responses accompanying behavioral expression of a CTA and mediated by the cervical vagus or spinal visceral afferents in the induction of c-FLI in the iNTS. Furthermore, rats in this experiment received three pairings of sucrose and LiCl, which resulted in a complete rejection of an intraoral infusion of sucrose. It is possible that vagotomy might attenuate either the behavioral expression or the correlated c-FLI induction in the iNTS in rats with weaker CTAs.

The aim of this experiment was to determine the role of the vagus in behavioral and neuronal expression of a CTA in rats that had acquired a CTA prior to surgery. There-

Fig. 2. Examples of c-FLI in the iNTS of sham-vagotomized rats (A–D) or vagotomized (E–H) rats with CTAs acquired against sucrose. Panels on right are higher-magnification images of areas outlined in left-hand panels. Few c-FLI-positive cells were seen in the iNTS of sham-vagotomized rats (A, B) sacrificed without treatment; intraoral infusion of sucrose induced c-FLI in the medial iNTS (C, D: black arrow). Numerous c-FLI-positive cells were present in the lateral iNTS of vagotomized rats without treatment (E) or 1 h after intraoral infusion of sucrose (G); c-FLI was present in the medial iNTS of vagotomized rats, however, only after intraoral infusion of sucrose (G, H). Black arrows outside the rectangle indicate c-FLI present in lateral NTS and centralis subnucleus of vagotomized rats. st, solitary tract; med, medial nucleus of the solitary tract; ce, centralis subnucleus; IV, fourth ventricle.

fore, all rats were conditioned against intraoral infusions of sucrose prior to vagotomy or sham-vagotomy. No unconditioned or non-contingent control groups were included, because we [12,14] and others [25,29,30] have established that c-FLI-induction in the iNTS by an intraoral infusion requires prior contingent pairing of the taste and toxin. The role of the vagus during acquisition of a CTA against intraoral infusions and the acquisition of the altered response of the iNTS correlated with CTA expression remains to be determined.

An unexpected and novel finding was that vagotomy itself caused c-FLI expression in a large numbers of cells in the centralis, subpostremal, and lateral regions of the NTS 1 week after surgery. While it has been reported that acute abdominal vagal stimulation or cervical vagotomy [24] induces c-FLI in the NTS within hours after surgery, several other published studies on vagotomized rats do not report c-FLI expression in the NTS at later time points [5,6,22,31]. The complete time course and mechanism of c-FLI induction in the NTS after vagotomy remain to be delineated.

Despite the profound response of some regions of the NTS to vagotomy, however, the specific response of the iNTS to the intraoral infusion of the conditioned taste stimulus was clearly distinguishable. Thus the c-FLI induction correlated with CTA expression appears anatomically and functionally independent of the effects of vagotomy on more lateral and caudal regions of the NTS. To demonstrate the independence of the vagotomy-induced c-FLI from the c-FLI induced after CTA expression, non-infused control groups were included.

In this study, we have excluded the subdiaphragmatic vagus as the neural pathway mediating c-FLI induction in the NTS correlated with CTA expression. This also eliminates conditioned gastrointestinal responses mediated and detected by the abdominal vagus as the proximal cause of the c-FLI induction. We and others have shown that the orofacial movements of rejection elicited by quinine are not sufficient to induce c-FLI in the iNTS [12,30]; therefore the orofacial rejection responses observed during CTA expression probably are not responsible for c-FLI induction in the iNTS.

Thus two of the major components of CTA expression, the orofacial motor output and vagally-mediated conditioned gastrointestinal responses, are not responsible for c-FLI induction in the iNTS correlated with CTA expression. The role of two categories of neural connections of the NTS remain to be tested. First, the induction of c-FLI in the iNTS during CTA expression may be dependent on other conditioned neural, humoral or motor responses mediated peripherally or in the caudal medulla. Second, the c-FLI in the iNTS may be dependent on activation of brain regions rostral to the NTS. Forebrain connections appear to be necessary because chronically decerebrate are not capable of CTA acquisition or expression [7]. Furthermore, Schafe et al. [25] have recently shown that hemidecerebra-

tion blocks c-Fos expression in the iNTS after CTA expression. We have recently found that c-FLI is induced in the central nucleus of the amygdala after CTA expression in a manner parallel to the iNTS [8]. Because the central nucleus has dense reciprocal connections with the NTS [15,21,23,26], this result suggests a causal link between activation of a specific forebrain region and activation of the iNTS during CTA expression. Preliminary findings that unilateral amygdala lesions attenuate the induction of c-FLI in the iNTS unilaterally after CTA expression support this hypothesis [10].

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