Functional vagal input to gastric myenteric plexus as assessed by vagal stimulation-induced Fos expression

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Zheng, Huiyuan, and Hans-Rudolf Berthoud. Functional vagal input to gastric myenteric plexus as assessed by vagal stimulation-induced Fos expression. Am J Physiol Gastrointest Liver Physiol 279: G73–G81, 2000.—Immunohistochemical detection of c-Fos expression was used to identify gastric myenteric plexus neurons that receive excitatory input from vagal efferent neurons activated by electrical stimulation of the cervical vagi in anesthetized rats. Vagal stimulation-induced Fos expression increased with higher pulse frequency, so that with 16 Hz (rectangular pulses of 1 mA/0.5 ms for 30 min) ~30% and with 48 Hz 90% of all neurons near the lesser curvature were Fos positive. In sham-stimulated rats there was no Fos expression. The percentage of Fos-activated neurons was only slightly smaller (85% with 48 Hz) near the greater curvature. Prior atropine administration (1 mg/kg ip) had little effect on vagal stimulation-induced Fos expression, and in unilaterally stimulated rats there was no Fos expression on the contralateral (noninnervated) side of the stomach, ruling out mediation by gastric motility or secretory responses. However, polysynaptic recruitment of third- and higher-order neurons cannot be ruled out completely. These results support the idea that, at least in the stomach, functional excitatory innervation of myenteric plexus neurons by the efferent vagus is sparse and widespread, refuting the notion of only a few vagal "command neurons." Vagal efferent outflow; vagal preganglionics; electrical stimulation; c-Fos; atropine; frequency dependency; fundus; corpus

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THE EFFERENT VAGUS NERVE is the major link between brain and stomach and is thought to be involved in important gastric functions such as gastric accommodation and relaxation (22, 30, 37), generation of phasic gastric contractions (8), and secretion of gastrin and gastric acid (35). Although these vagally dependent gastric responses have been extensively studied at the physiological output level, the exact mechanisms and the particular enteric neurons involved are not well understood. Little is known about the specificity of vagal responses and the neurochemical and morphological characteristics of both the preganglionic efferent vagal and enteric neurons involved. Recently emerging evidence suggests that vagal outflow to the gastrointestinal tract may be organized more discretely than assumed by the more traditional views of autonomic outflow and parasympathetic tone (14, 19, 30, 36, 40).

In the early 19th century, when autonomic neurophysiology was established, Langley (20) concluded that the relatively few vagal preganglionic neurons innervate only a small proportion of the much more numerous enteric neurons, a concept termed the "command neuron" hypothesis. If this hypothesis were correct, it would be of great interest to know what distinguishes the "command neurons" from the other enteric neurons in terms of their location, neurochemistry, and projection pattern. However, more recently it became evident that the hypothesis may apply only to the small and large intestines and not to the stomach. Anterograde neuronal tracing with the carbocyanine dyes Dil and DiA (2–4, 18) or Phaseolus vulgaris leucoagglutinin (12) injected into the vagal dorsal motor nucleus revealed an extremely dense innervation pattern in the rat stomach, with a large proportion of enteric neurons in the myenteric plexus receiving close anatomic contacts from varicose vagal efferent axon terminals. Although it is difficult to infer synaptic input on the basis of anatomic contacts, these findings were essentially confirmed by an electrophysiological study in the vagally innervated guinea pig stomach (32). Using intracellular recording, these authors found that near the lesser curvature of the stomach almost all neurons could be synthetically activated by electrical stimulation of the vagal supply and that the percentage of activated neurons dropped off considerably toward the greater curvature. By combining the anterograde tracing method with NADPH histochemistry and peptide immunohistochemistry, we have demonstrated that vagal preganglionic axon terminals specifically contact nitric oxide- and gastrin-releasing peptide-producing neurons in the gastric myenteric plexus (2). It was clear, however, that vagal preganglionic input was not restricted to neurons with a particular neurochemical phenotype, and it can be difficult to assume synaptic input on the basis of close anatomic contact (2).

An alternative method to identify neural connectivity is the immunohistochemical detection of immediate-early genes and their protein products such as c-Fos.
Although this method has been used extensively in the central nervous system, its usefulness as an activity marker in the enteric nervous system has only recently been described (27, 29, 34). It appears that most enteric neurons, including intrinsic sensory neurons, have the capacity to express c-Fos (17, 26). The absence of spontaneous Fos expression, particularly in the stomach, was a prerequisite for this approach (39). Furthermore, on the basis of experiments in rats with degenerated vagal afferent fibers after supranodose vagotomy, antidromic activation of vagal afferent fibers induces Fos in only a very small number of enteric neurons (39).

Therefore, the aim of the present study was to identify enteric neurons in the rat stomach that receive vagal afferent input, by virtue of their c-Fos expression on electrical stimulation of the vagus nerve. This method should provide a more functional assessment of vagal input and should overcome some of the interpretational problems with the purely anatomic method of anterograde tracing. It would also be amenable to combination with immunohistochemistry to determine the neurochemical phenotype of vagally addressed enteric neurons in future experiments.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 200–300 g (Harlan Industries, Indianapolis, IN) were housed individually in hanging wire mesh cages under standard laboratory conditions (12:12-h lighting schedule, lights on at 700, 22 ± 3°C) with 5001 Purina lab chow and tap water available ad libitum.

Electrical stimulation and control procedures. Rats were anesthetized between 900 and 1300 with pentobarbital sodium (65 mg/kg ip) or ketamine-acetophenazine-xylazine (100, 2, and 5 mg/kg, respectively, sc) and placed on a regulated (38°C) heating pad. Some rats (see Experimental groups) were given injections of atropine (1 mg/kg ip). A piece of PE-90 tubing was then inserted into the cut trachea to ease respiration. The left cervical vagus was carefully isolated and freed from all connective tissue for a length of ~15 mm and ligated centrally. The nerve was then placed on bipolar platinum hook electrodes and covered with prewarmed mineral oil. After a resting period of 10 min, 30-min electrical stimulation with various frequencies, or sham stimulation, was started. Electrical stimulation consisted of rectangular pulses of 1-mA intensity and 0.5-ms duration (measured as voltage drop across an in-series resistor and an oscilloscope) and stimulation frequencies of 6, 16, 24, and 48 Hz. Sham or control stimulation consisted of the same initial surgical procedure. The cervical vagus was then cut 10 mm distal to the stimulation electrode, and stimulation was carried out at 48 Hz to control for all possible effects associated with the procedure, including motor responses of the esophagus or adjacent neck musculature occurring in some animals. The maximal effectiveness of the current intensity (1 mA) and pulse duration (0.5 ms) was determined in a similar paradigm using Fos expression in neurons of pancreatic ganglia as end point.

At the end of the 30-min stimulation period the electrode was removed, the cervical wound was closed with wound clips, and the animals were left untouched for another 60 min before euthanasia and perfusion.

Experimental groups. In experiment 1, the effect of stimulation frequency was investigated. A total of 24 rats anesthetized with pentobarbital sodium (65 mg/kg ip) and treated with atropine (1 mg/kg ip) 15 min before the start of stimulation were randomly assigned to stimulation with the control procedure or with 6, 16, 24, or 48 Hz (1 mA, 0.5 ms, 30 min).

In experiment 2, the effect of atropine on vagal stimulation-induced Fos expression was tested. A total of 24 rats anesthetized with either pentobarbital sodium (n = 12) or ketamine-xylazine-acetophenazine (n = 12) were stimulated with 16 Hz (1 mA, 0.5 ms, 30 min) in the fasting state. Sections (20–25 mm thick) were removed, the cervical wound was closed with wound clips, and the animals were left untouched for another 60 min before euthanasia and perfusion.

In experiment 3, the effect of different anesthetics was tested. In our first experiments with pentobarbital sodium anesthesia, we found no Fos expression in the stomach in sham-stimulated rats. However, the presence of Fos expression in the small intestine prompted us to look for another mode of anesthesia, which we found in the ketamine-xylazine mixture. Therefore, a total of 22 rats treated with atropine and anesthetized with either pentobarbital sodium (n = 12) or ketamine (n = 10) were stimulated with either 16 (n = 6) or 48 (n = 4–6) Hz. Some of the rats also participated in experiments 1 and 2.

In experiment 4, the effect of local fundic vagal denervation was evaluated. Two rats were anesthetized with ketamine-xylazine as above. The major fundic vagal branch originating from the main ventral gastric vagal trunk was isolated from the trachea and cut with a high-temperature tissue cautizer. The wound was closed, and the rats were allowed to recover for 10 days. The left cervical vagus was then stimulated as in the other animals.

C-Fos immunohistochemistry and neuronal counterstain. One hour after termination of stimulation, rats were euthanized with a lethal dose of pentobarbital sodium, and when fully unresponsive they were transcardially perfused with 200 ml of heparinized (20 U/ml) saline followed by 500 ml of cold phosphate-buffered (pH 7.4) 4% paraformaldehyde. The entire gut was then extracted and divided into esophagus, stomach, duodenum, jejunum/ileum, cecum, colon, and pancreas. The stomach was opened at the greater curvature, thoroughly rinsed, and postfixed for 24–48 h with the same fixative. Before cutting, tissue samples were blocked and immersed for 24 h in 25% sucrose in PBS for cryoprotection. The tissue blocks were then mounted on a chuck using 1 ml of Tissue-Tek O.C.T. embedding medium and quickly frozen. Sections (20–25 mm thick) were cut in a cryostat and collected free-floating in PBS. The method for neuronal counterstain was adapted from Holst and Powley (13). Briefly, sections were first treated with hydrogen peroxide (3% H2O2 and methanol, 1:4) for 20 min at room temperature followed by three washes with PBS. After a brief rinse in distilled water the sections were stained for 2 h at 42°C in cuproin blue (0.3% quinolinic phthalocyanine in 0.05 M sodium acetate-1.0 M magnesium chloride buffer, pH 4.9). They were then rinsed briefly in distilled H2O, differentiated for 2 min in the above buffer, and finally washed three times in PBS. For subsequent c-Fos staining the sections were immersed in a blocking solution (5% nonfat milk, 10% normal goat serum, 1% BSA, 0.5% Triton X-100) for 1 h before incubation with the rabbit c-Fos primary antibody (AB-5, Oncogene; 1:25,000 dilution) for 40–60 h at 7°C. The sections were rinsed at 4°C in PBS-Gel (0.1% gelatin) and then incubated for 90 min in biotinylated secondary antibody (Jackson Immunoresearch; 1:100 dilution). After three rinses in PBS-Gel, the sections were incubated for 1 h in an avidin-biotin complex (1:50, Vectastain ABC Elite Kit, Vector Laboratories). The antibody complex was then rinsed again three times in PBS and visualized using a metal-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB) Substrate Kit (Pierce Chemical, Rockford, IL) for 3–5 min, resulting in a dark blue-black nuclear stain.
As a test for specificity of the c-Fos antibody, a few sections from the corpus were processed the same way except that incubation in the primary c-Fos antibody was omitted. This resulted in complete absence of any staining (see Fig. 2E).

Sampling strategy and counting procedure. Samples of stomach wall (10 mm) were taken from the stimulated side (ventral) of the stomach. Three locations of corpus with respect to the curvatures (near the lesser curvature or cardia, near the greater curvature, and from the middle third) and one location of fundus (middle third) were sampled for each rat. Each sample thus yielded a number of sections containing areas of the myenteric plexus. For determination of the proportion of activated neurons, a total of at least 500 neurons were inspected from randomly selected sections from a given sample. Neurons were recognized by their turquoise cuprolinic blue stain, and only neurons with a clearly visible nucleus were considered. For each neuron counted, the determination was made as to whether its nucleus was stained for Fos. Staining intensity of Fos-positive neuronal nuclei ranged from light brown to black and was always distinguishable from the absence of stain in Fos-negative neurons.

To ensure that Fos induction in the myenteric plexus neurons was the direct result of synaptic activation via preganglionic nerve terminals, the contralateral or nonstimulated side of the stomach was also examined in some rats and used as an internal control for indirect Fos induction possibly due to vagally induced changes in gastric motility, hormones such as gastrin or insulin, or local chemical changes such as mucosal acidification. With both anterograde and retrograde tracing strategies, it has been shown that in the rat the ventral (left) and dorsal (right) vagal trunks almost exclusively innervate the ventral and dorsal side of the stomach, respectively (Fig. 1).

Statistical analysis. The SAS Mixed Procedure was used on the percentage of neurons expressing Fos to generate a repeated-measures ANOVA. The repeated-measures model contained one within-subject main effect (location), three between-subject main effects (frequency, atropine/saline pre-treatment, and type of anesthesia), and all possible interaction effects. Test-of-effect slices provided a one-way ANOVA on the effect of a variable of interest while holding the other three variables in the model constant at specified levels. The Bonferroni adjustments to the p values in the post hoc comparisons were designed to hold the overall type I error for each question to <5%.

RESULTS

Most importantly, there was no significant spontaneous Fos expression anywhere in the stomach myenteric plexus after sham stimulation under either pentobarbital sodium or ketamine anesthesia or in the presence or absence of atropine (±0.68 ± 0.6% for any given location and condition). In 26 of 33 rats with sham stimulation not one single Fos-positive neuron was detected. In contrast, real stimulation of the intact vagus nerve produced widespread immunohistochemical staining of Fos protein visible as a light to dark brown DAB reaction product mostly limited to neuronal cell nuclei (Fig. 1). Background stain typically consisted of a faint yellow to light brown stain in nerve fibers of the myenteric plexus. Both nuclear and background stain were absent in tissue processed without incubation in primary antibody (Fig. 2E). Neurons were counted as Fos positive if their nucleus was stained at an intensity that was clearly above the faint background stain. Neurons were counted as Fos negative if their nucleus was contained within the section and was either not stained at all or stained near the intensity of background staining of the surrounding myenteric plexus strands. Neurons were identified by virtue of their turquoise cuprolinic blue stain (Fig. 1).

Effect of varying frequency of stimulation. The frequency-response relationship for the expression of Fos was characterized by a complete absence of Fos activation with control stimulation, a near 90% recruitment with 48 Hz, and a half-maximal response with ~20 Hz (Figs. 1–3). The main effect of frequency was highly significant and almost identical for the gastric fundus and corpus [corpus: F (4,48) = 145.7, P < 0.001; fundus: F (4,48) = 82.0, P < 0.01].

Lesser-to-greater curvature gradient. Local differences in Fos expression in the corpus myenteric plexus were assessed in two groups of rats with 16 (n = 6) and 48 (n = 6)-Hz stimulation, respectively, in the presence of atropine and under pentobarbital sodium anesthesia. With a stimulation frequency of 16 Hz there was no significant difference in neuronal Fos expression between tissue samples taken near the lesser or greater curvatures or from the middle portion of the stomach wall [F (3,48) = 1.1, P = 0.36] (Fig. 4). There was also no regional difference with a 16-Hz stimulation in the absence of atropine (results not shown). 48-Hz stimulation, the proportion of neurons expressing Fos was slightly but significantly less near the greater curvature compared with the lesser curvature [Fig. 4; t (48) = 3.77, P < 0.01]. The difference between the lesser curvature and the middle portion and between the middle portion and the greater curvature was, however, not significantly different.

Effect of atropine pretreatment. The effect of cholinergic blockade with atropine was compared in four groups of rats stimulated with 16 Hz and anesthetized with either pentobarbital sodium (n = 4–6) or ketamine (n = 6–7). Although atropine only slightly but nonsignificantly decreased the proportion of Fos-positive neurons in animals under ketamine anesthesia [F (1,48) = 3.73, P = 0.36], it slightly but nonsignificantly increased Fos expression in animals under pentobarbital sodium anesthesia [Fig. 5; F (1,48) = 2.38, P = 0.78].

Absence of Fos expression in myenteric plexus neurons not directly vagally innervated. In two groups of rats with 16- and 48-Hz unilateral stimulation, respectively, and in the presence of atropine, the proportion of Fos-positive neurons was determined both on the ipsilateral (innervated) and contralateral (noninnervated) side of the gastric corpus and fundus. Although there was full Fos expression on the ipsilateral side, there was virtually no Fos expression on the contralateral side of the same stomach [Fig. 6A; t (4) = 20.6, P < 0.01].

In two animals, the ventral fundic branch of the ventral vagal trunk was cauterized 10 days before stimulation of the left cervical vagus with 48 Hz. Although full Fos expression (89.3 ± 2.9%) was found in samples from the ventral corpus, only a few Fos-
positive neurons (6.1 ± 3.5%) were found in samples from the ventral fundus, supplied by the fundic branch (Figs. 1D and 6B). Effect of different anesthetics. The effects of pentobarbital sodium and ketamine anesthesia were assessed in rats stimulated with sham stimulation (n = 4–7) or 16 (n = 6) or 48 (n = 4–6) Hz in the presence of atropine (Fig. 7). As mentioned above, sham stimulation did not induce any Fos expression under either anesthesia. With 16-Hz stimulation, there was no difference in Fos expression between the two anesthetics except for a slightly but significantly higher expression in the middle part of the corpus with ketamine [F(1,48) = 8.27, P < 0.05]. With 48-Hz stimulation, significantly less Fos was expressed at all three locations of the gastric corpus in ketamine-anesthetized rats [F(1,48) = 8.1–18.6; all P < 0.05].

DISCUSSION

With supramaximal stimulus parameters at 48 Hz, unilateral stimulation of the cut peripheral vagi induced Fos expression in ~90% of ipsilateral but not contralateral gastric myenteric plexus neurons. These results suggest that most enteric neurons in the rat gastric myenteric plexus receive functional input from vagal efferent neurons and that this input is strictly lateralized. The basic finding corroborates earlier purely anatomic findings with anterograde tracing of vagal preganglionics in rats (3, 12) and electrophysiological data with intracellular recording from enteric neurons in the guinea pig (32). Before we discuss some functional implications of such widespread vagal input, we first consider the possibility that Fos expression might result from indirect activation of enteric neurons.

Because with sham stimulation there was no spontaneous Fos expression in neurons of the rat gastric myenteric plexus, any Fos expression with vagal stimulation must be the result of direct or indirect effects of such stimulation. As direct effects we define synaptic inputs from vagal preganglionic efferent axon terminals to enteric neurons. As indirect effects we can consider mechanisms 1) via antidromic stimulation of vagal afferent fibers, 2) via gastric motility and secretory responses and enteric sensory neurons, 3) via the release of hormones and other factors that activate enteric neurons via paracrine or true hormonal mecha-
nisms, 4) via changes in cardiovascular and respiratory parameters, and finally 5) via enteric interneurons (3rd- or higher-order neuron recruitment). Electrical stimulation of the cervical vagus nerves inadvertently leads to an antidromic activation of vagal afferent fibers. This could result in transmitter release from vagal afferent terminals in the myenteric plexus, the so-called intraganglionic laminar endings (IGLEs) and, in turn, induction of Fos in surrounding enteric neurons. That this is not the case has been recently shown in experiments with intentional electrical stimulation (at 16 Hz) of vagal afferent fibers after vagal efferent fibers had been eliminated by prior supranodose vagotomy (39). We have also confirmed this absence of significant Fos expression with stimulation at

![Photomicrographs of flat sections of gastric wall showing nuclear c-Fos labeling (black) in neurons of myenteric plexus, induced by electrical stimulation of cervical vagus nerve in anesthetized rats. Digital images generated in transmitted light mode with laser scanning microscope using 633 nm HeNe laser line are shown. A and B: low- and high-power images, respectively, from ipsilateral (stimulated) side of corpus (near lesser curvature) of rat stimulated with optimal frequency of 48 Hz in the presence of atropine. Note that most of the neurons express Fos. C and D: low- and high-power images, respectively, from ipsilateral side of corpus (middle part) of rat stimulated with 16 Hz. Note clustering of Fos-expressing neurons in C. E: negative control experiment from rat with 48-Hz stimulation but omission of primary antibody. Note the complete absence of nuclear Fos stain and absence of light stain in neuropil and interconnective strands, as seen in other panels. Contrast and sharpness have been electronically adjusted to show optimal staining differences. Scale bar: 150 (A), 20 (B and D), 70 (C), 60 (E) µm.

![Frequency-response relationship for vagal stimulation-induced Fos expression in myenteric plexus neurons of gastric fundus and corpus in pentobarbital-anesthetized, atropine-pretreated rats. The number of Fos-positive neurons is expressed as a percentage of the total number of neurons as assessed by cuprolinic blue counterstain. Means ± SE of 4–6 animals are shown. Bars that do not share the same superscripted letter are significantly (P < 0.05) different from each other (based on ANOVA followed by post hoc Bonferroni-adjusted multiple comparisons).

![Lesser-to-greater curvature gradient of vagal stimulation-induced Fos expression in rat gastric corpus shown for atropine-treated, pentobarbital-anesthetized rats and 2 stimulation frequencies. Note that although there was no gradient with 16 Hz stimulation, there was slightly but significantly less Fos expression near the greater curvature compared with the lesser curvature with 48-Hz stimulation. Means ± SE of 5–6 rats are shown. Bars that do not share the same superscripted letter are significantly different (P < 0.05, based on post hoc Bonferroni-adjusted multiple-comparison test after ANOVA).
48 Hz (unpublished observations). Less than 1% of gastric myenteric plexus neurons could be induced to express Fos in such animals, suggesting that excitatory synaptic input via vagal afferent axon collaterals in the form of IGLEs is unlikely. However, we cannot rule out the possibility that transmitter released from afferent nerve terminals might inhibit myenteric neurons or modulate their excitability.

Fos expression induced via gastric motility and secretory responses and sensory enteric neurons is highly unlikely, because most of the phasic gastric contractions and gastric acid secretion induced by vagal stimulation are muscarinic and thus inhibited by atropine. It has been shown that atropine at the dose level used in the present experiment suppressed electrical vagal stimulation-induced (16 Hz) gastric acid secretion in anesthetized rats by 70% (33), and it typically completely abolished gastric motility and secretory responses induced by vagally mediated chemical brain stimulation (9, 24). Although we have not determined whether atropine at the dose level used could block gastric motility and acid secretion induced by 48-Hz electrical stimulation, it must have greatly reduced these. Because atropine did not significantly decrease Fos expression such responses are unlikely to be mediators of Fos expression. Furthermore, it could be expected that strong gastric contractions or luminal acidification propagates to the contralateral side of the stomach and induces Fos expression there, but Fos expression was strictly limited to the ipsilateral side of stimulation. Similarly, Fos expression induced via the release of hormones, or via changes in cardiovascular and respiratory parameters, is also highly unlikely because these effects would not be confined to the ipsilateral side of the stomach. Finally, although our local denervation control does not rule out mediation by very localized paracrine effects, it does argue against hormones and other factors acting in a semilocal fashion as mediators of the Fos expression.

Generally, enteric interneurons transmit signals between sensory and motor neurons and between different populations of motor neurons (7). Therefore, they could spread excitation beyond the neurons receiving direct vagal input. This could result in a high percentage of Fos-positive neurons despite only relatively few enteric neurons receiving direct vagal input consistent
with the command neuron hypothesis (16, 20). With the present data we cannot rule out some degree of such spread of excitation, but there are several arguments that can be made against it.

First, the observation by Schemann and Grundy (32) that neurons within a given ganglion were all activated after the same latency suggested that vagal input to most neurons is monosynaptic. Second, Fos expression was almost completely absent from a restricted area in two rats with surgical transection of the major fundic vagal branch. Myenteric plexus neurons in this area most certainly receive excitatory inputs from orally projecting interneurons (5) located up to 20 mm aboral and outside the vagally denervated area, and one could therefore expect spread of vagal activation via these interneurons.

Third, although there is limited involvement of muscarinic cholinergic transmission in the enteric nervous system, atropine treatment could have been expected to block at least some of the Fos expression via polysynaptic pathways. However, it only marginally reduced Fos expression and only with pentobarbital sodium anesthesia and not with ketamine anesthesia. The bulk of excitatory neural transmission in the myenteric plexus is thought to be accomplished by other than muscarinic cholinergic transmission, such as nicotinic cholinergic (31), substance P (38) via neurokinin NK-1 and NK-3 receptors (15), ATP (10), and glutamate (21). The effect of vagal stimulation on enteric interneuronal activity mediated by these excitatory transmitters must be investigated with the Fos method or with electrophysiological techniques.

On the other hand, at least two findings of our study may indicate support for multisynaptic enteric neuron recruitment. Near the greater curvature we found ~80% vagally activated neurons, whereas in the electrophysiological study of Schemann and Grundy (32) only ~20–30% of neurons could be activated. Besides being a species difference, this discrepancy could indicate spread of excitation and induction of Fos beyond vagal postganglionic neurons. Also, Fos expression was significantly lower in ketamine compared with pentobarbital sodium-anesthetized animals. Ketamine is known to act as an antagonist for the glutamatergic N-methyl-D-aspartate (NMDA) receptor. Neurons containing glutamate (21) and neurons bearing glutamatergic NMDA receptors (6) have been identified in the enteric nervous system. If this transmitter system is indeed responsible for some of the excitatory neurotransmission between enteric neurons, ketamine could thus block some of this secondary activation and result in less Fos expression.

Thus, although the arguments against widespread third- or higher-order neuron recruitment via polysynaptic pathways seem stronger, additional experiments are necessary to obtain a definitive answer. One approach will be the pharmacological blockade of candidate excitatory transmitter actions during vagal stimulation. An alternative approach may combine anterograde tracing from the dorsal motor nucleus with subsequent stimulation-induced Fos expression and analysis of the correlation between terminal structures around, and Fos expression in, a given enteric neuron.

No matter what the degree of polysynaptic activation might be, the present results demonstrate that most, if not all, neurons in the gastric myenteric plexus receive excitatory input from vagal preganglionic nerve terminals. This finding underscores the many observations demonstrating a strong modulation of gastric functions by psychological moods and factors such as anger and stress. It will be important to investigate further the extent of functionally specific vagal efferent pathways and the neurotransmitters and receptor types involved.

In the present experiment we have not attempted to block vagal stimulation-induced Fos expression with a ganglionic blocker such as hexamethonium. In our previous report (39), a total dose of 30 mg/kg of this blocker did not completely suppress, but substantially reduced by ~65%, Fos expression induced by vagal stimulation at 16 Hz. Incomplete suppression of vagal stimulation-induced gastric responses in the rat has also been reported by other investigators (33) and may simply indicate the difficulty of obtaining a high-enough local concentration at the relevant synapses in the in vivo situation. Alternatively, it may suggest the release of noncholinergic cotransmitters by vagal efferent axon terminals. This possibility is further discussed in the following section.

Perspectives

The aim of the present study was to activate all vagal efferent fibers with supramaximal stimulation parameters to assess the quantitative aspect of gastric vagal innervation. Because the innervation is so widespread, the conclusion is that all classes of gastric enteric neurons receive functional input. However, this approach does not address the issue of target specificity of functionally distinct populations of vagal efferent neurons. To answer this question selective stimulation of only certain populations of vagal efferent fibers would be necessary. The pioneering work of Martinson (23) resulted in the distinction of at least two populations of vagal efferents: low- and high-threshold fibers. Low-threshold fibers can be activated with low-voltage electrical stimulation, correspond to medium-size unmyelinated or slightly myelinated fibers, and produce mainly muscarinic cholinergic gastric contractions. High-threshold fibers need a higher voltage/current for activation, correspond to very thin unmyelinated fibers, and are responsible for gastric acid secretion and noncholinergic, nonadrenergic smooth muscle inhibitory responses. The very thin high-threshold fibers have difficulties in following high frequencies because of axon block. To successfully activate all fiber types, we first used a combination of high stimulus strength (as determined by the current and pulse duration) and relatively low frequencies of 6 and 16 Hz to prevent axon block. It was surprising that these parameters produced limited Fos expression and that a frequency of 48 Hz was necessary to obtain maximal Fos expression. How could this apparent discrepancy be explained? It could simply mean that in most enteric
neurons high frequency is necessary to induce Fos, whereas physiological responses such as gastric acid secretion require lower frequencies. However, the higher frequency for Fos expression may not necessarily be termed “unphysiological.” In the sympathetic nervous system it has become clear that there is a preferential release of certain cotransmitters with high-frequency stimulation (28). Although this has not yet been demonstrated for vagal efferent preganglionic neurons, some of them have been found to produce other potential transmitters such as nitric oxide, ATP, dopamine, and galanin, and some responses to vagal stimulation could not be completely blocked by hexamethonium (33, 39).

It is also interesting that the natural firing pattern of vagal efferent neurons includes both low- and high-frequency components in that instantaneous frequency, based on the occurrence of spike intervals, can reach maxima well above 50 Hz (Ref. 25; D. Adelson, personal communication). It is intriguing to speculate that vagal efferent axon terminals may, therefore, be able to communicate information to enteric neurons by more than one channel.

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REFERENCES