# Brainstem Origins of Pyloric Preganglionic Parasympathetic Vagal Neurons in the Ferret (*Mustela Putorius furo*) using the Retrograde HRP Transport Neurohistochemical Technique

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## ABSTRACT

The brainstem origins of vagal preganglionic parasympathetic neurons innervating the pylorus were studied in the ferret using the horseradish peroxidase (HRP) neurohistochemical technique. A total of 22 adult male and female ferrets (16 experimental and 6 controls) were used for the study. In eight of the experimental ferrets, both the pyloric canal and antrum were injected simultaneously by multiple penetrations with 0.1 ml of 23% HRP (Sigma type VI) in normal saline. In four of the experimental ferrets, only the pyloric canal was injected with HRP while in another four of the experimental ferrets, only the pyloric antrum was injected with the tracer. The control ferrets were injected in a similar manner as the experimental ferrets with normal saline. After 48-72 hours of post-injection survival period, each ferret was perfused transcardially, first with normal saline followed by buffered fixative and finally with buffered sucrose. Craniotomy was performed and the brainstem excised from the brain and spinal cord. Transverse serial frozen sections of the brainstem were taken and processed for HRP neurohistochemistry and then analyzed under light and dark-field microscopy. The results of the study showed that in the experimental ferrets, neurons of the dorsal motor nucleus of the vagus nerve (DMNV) as well as those of the nucleus ambiguus (nA) were labeled with HRP. The results of the experiment also showed that the nA projects only to the pylonic canal. The control ferrets did not reveal any HRP labeled neurons in the brainstem.

Keywords: Dual vagal nuclear innervation, Ferret, Horseradish peroxidase, Pylorus.

## INTRODUCTION

Although the pylorus is often regarded anatomically as an integral part of the stomach, it is functionally and histologically distinct from the stomach [1,2]. A review of the literature shows that several studies have been carried out on the pylorus in man and other species [3,4-6]. Following these studies, it is now well established that the pylorus serves as an effective anatomical and physiological sphincter between the stomach and the duodenum.

The role of the pylorus in the complex duodenogastric reflux has also generated a lot of interest among investigators in view of the impact of these functions on the course and prognosis of gastroduodenal diseases [4,5].

As regards the control and modulation of pyloric functions, it has also been shown that pyloric vagal innervation consists of both excitatory and inhibitory fibers [7,8-11]. Furthermore, earlier studies have also revealed that in addition to the commonly known neurotransmitter, acetylcholine [12], other neurotransmitters, and modulators, including encephalin [13] vasoactive intestinal peptide

(VIP) [14,15], substance P [16]; and peptidergic hormones [17] have also been shown to be associated with vagal functions at the pyloric terminals. There are speculations that some of these neurotransmitters may be implicated in stimulating secretion of gastrointestinal hormones and other substances, which are involved in pyloric as well as gastric functions [18,19].

An earlier suggestion of the existence of a true anatomical sphincter at the gastroduodenal junction by Horton [20] has also been confirmed by detailed comparative studies of the pylorus in man and other mammals including the rabbit, cat, dog, pig, ox and horse [3]. Physiological sphincteric characteristics have also been shown to exist in the pylorus [21,22].

In spite of these extensive studies on the functions and control of the pylorus, there are limited studies on the origins of vagal neurons supplying the pylorus [6,23-28] .

Although the ferret has been shown to be a very suitable model for experimental studies, particularly of the gastrointestinal system [29- 34], there are no records as yet of the origins of vagal fibres innervating this segment of the gastrointestinal tract in the ferret.

The present paper is a report of our investigations of the vagal innervation of the pylorus in the ferret using the HRP neurohistochemical technique.

## MATERIALS AND METHODS

A total of 22 male and female adult ferrets, weighing between 800 and 1500 g were used for the study. All the ferrets were kept in a well-ventilated and illuminated animal house. Each ferret was kept in a separate cage to which was fitted a water bottle with a special dispenser which allows the ferret free access to water. They were all fed with ferret pellets (supplied by the animal house) to which they also had free access.

For surgical exposure of the pylorus, each ferret was anesthetized with an intraperitoneal injection of sodium pentobarbitone (supplied by Sagatal, May and Baker, Dabenham) in a dose of 60 mg/kg body weight. A midline laparotomy was then performed and the pylorus and the adjoining parts of the stomach and the duodenum delivered to the anterior abdominal wall.

With the aid of a 100 microliter Hamilton syringe and needle, 0.1 ml of 23% Horseradish peroxidase (Sigma type VI) in normal saline was injected by multiple penetrations into the muscular coat of the pyloric antrum and canal up to the pyloroduodenal junction (Fig. 1). A total of eight ferrets were injected in this manner.

In four ferrets, the pyloric canal only was injected while in four other ferrets, only the pyloric antrum was injected with the tracer. The eight ferrets in this group were injected with the same quality and percentage of HRP and in the same manner as the first group of eight ferrets. In all injections into the pyloric wall, the upper border (continuation of the lesser curvature into the pylorus) as well as the lower border (continuation of the greater curvature into the pylorus) was avoided in order to minimize the risk of labeling “fibers of passage” These are fibers passing along the lesser curvature but not supplying the stomach. The remaining six ferrets, which were used as controls, were divided into three sets. The first set of two ferrets was injected with 0.1 ml of normal saline in a similar manner as the experimental ferret after bilateral truncal vagotomy, while the last set of two ferrets had the same quantity and concentration of HRP injection into the peritoneal cavity (without laparotomy).

In each experiment, the needle was left in place at the injection site for about two minutes after injection in order to avoid leakage of HRP into the peritoneal cavity.

After HRP injections in each experiment, the laparotomy incision was closed in layers and the animals kept for a survival period. Each animal was perfused transcardially after anaesthesia, initially with normal saline followed by a fixative containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M-phosphate buffer sucrose at 4ºC and caudal to the obex with the highest concentration of labeled neurons occurring in the region of the obex.

The mean rostrocaudal extent of HRP labeled neurons was from 3.23 mm rostral to the obex to 1.63 mm caudal to the obex (Table 1). Furthermore, an average of 42.81% of the total neurons counted in sampled sections in the eight ferrets was labeled with HRP (Table 1.)

HRP labeled neurons were also seen in the nA of the first group of eight experimental ferrets (Figs 3 and 4). In this group, the distribution of labeled neurons in the nA was bilateral but sporadic and sparse when compared with the pattern of distribution of labeled neurons in the DMNV.

In the four ferrets in which only the pyloric canal was injected, HRP labeled neurons were seen in the DMNV as well as in the nA, while sections taken from the four ferrets which had injections into the pyloric antrum had HRP labeled neurons in the DMNV only.

On examination of the sections taken from the six control ferrets, none of the sections revealed labeled neurons in any of the brainstem nuclei, indicating that the HRP labeling seen in the experimental ferret were due to HRP injections into the pylorus. The results of the control ferrets also indicate that the vagus nerve is responsible for the neuronal transport as proposed by Mesulam [35,36]. The brainstems were then removed, sectioned serially at 20 μm thickness with a freezing microtome and processed for HRP neurohistochemistry using the tetramethylbenzidine method as recommended by Mesulam [35,36].

The sections were then analyzed under bright and dark-field illuminations. In analyzing the sections, cells with distinct margins and in which the blue reaction granules of TMB/HRP were seen in their cytoplasm, were regarded as HRP labeled cells. Whole cells with distinct margin but not containing any reaction granules were regarded as unlabeled cells. Cells counting was done from every fourth section of the brainstem in each of the first group of eight experimental ferrets using a calibrated microscope slide, as earlier described by Abercrombie [37].

## RESULTS

On examination of the sections taken from the first group of eight experimental ferrets, HRP-labeled neurons were seen in the DMNV (Figs. 2 and 4). The distribution of labeled neurones was bilateral and confined to the medial aspect of the nucleus. HRP labeled neurons were also distributed rostral and caudal to the obex with the highest concentration of labeled neurons occurring in the region of the obex.

The mean rostrocaudal extent of HRP labeled neurons was from 3.23 mm rostral to the obex to 1.63 mm caudal to the obex (Table 1). Furthermore, an average of 42.81% of the total neurons counted in sampled section sections in the eight ferrets was labeled with HRP (Table 1).

HRP labeled neurons were also seen in the nA of the first group of eight experimental ferrets (Figs. 3 & 4). In this group, the distribution of labeled neurons in the nA was bilateral but sporadic and sparse when compared

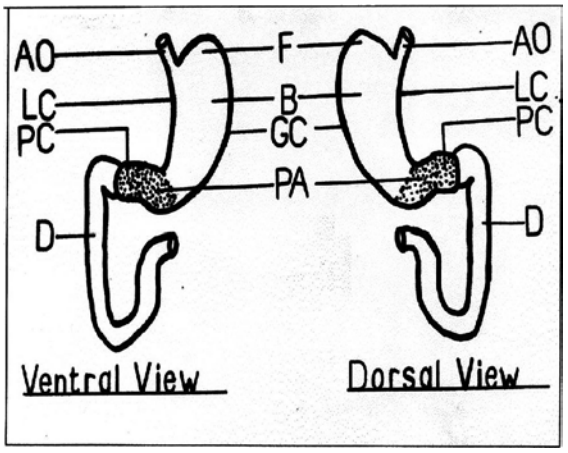


Fig. 1. Schematic representation of the pylorus and adjoining parts of the of the gastrointestinal tract. Dotted areas illustrate HRP injection sites.
AO = Abdominal esophagus
F = Fundus of the stomach
LC = Lesser curvature
B = Body of the stomach
GC = Greater curvature
PA = Pyloric antrum
PC = Pyloric canal
D = Duodenum

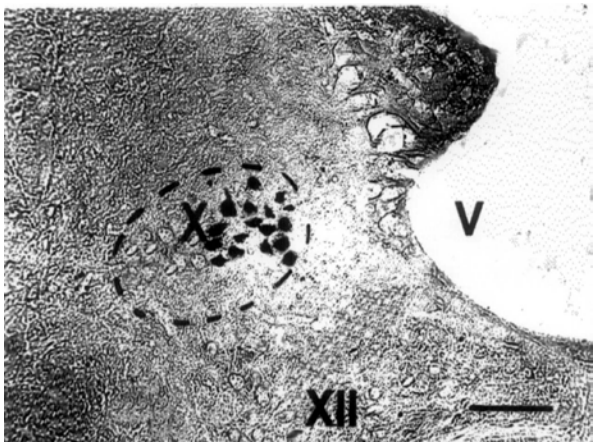


Fig. 2. Photomicrograph of a transverse section of the medulla oblongata rostral to the obex, showing HRP labeled neurons in the Dorsal motor nucleus of the vagus nerve (circumscribed in dotted lines) following HRP injection into the Pylorus (bar = 100 μ).

V = Fourth ventricle
X = Doral motor nucleus of the vagus nerve (DMNV), with dark-colored HRP labeled neurons .
XII = Hypoglossal nucleus

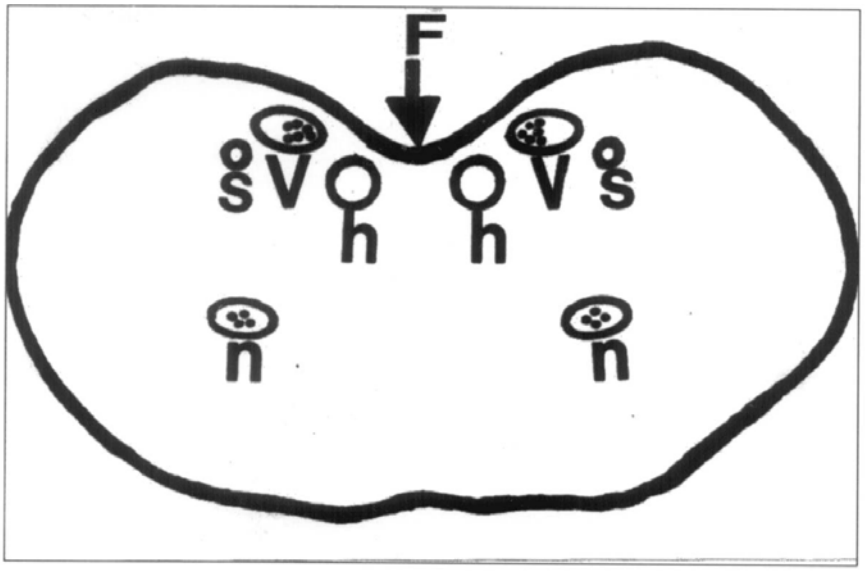


Fig. 3. Schematic representation of some brainstem nuclei in a transverse section through the medulla oblongata, rostral to the obex: Dotted areas illustrate localization of HRP labeled neurons, following HRP injection into the pylorus:

F = Floor of the fourth ventricle
h = Hypoglossal nucleus
V = Doral motor nucleus of the vagus nerve.
n = Nucleus ambiguus
s = Nucleus of tractus solitarius

**Table 1: Summary of analysis of cell counts in the dorsal motor nucleus of the vagus nerve and the nucleus ambiguus following HRP injections into the pylorus.**

| Experiment number | Injection site | Number of HRP labeled cells counted in DMNV | Total number of cells counted in the DMNV | Percentage of labeled to total number of cells (%) | Number of labeled cells counted in the nA | Rostrocaudal extent of labeled DMNV (mm) |
|---|---|---|---|---|---|---|
| 1 | Pylorus | 822 | 1901 | 43.24 | 21 | 3.60 to –1.52 |
| 2 | Pylorus | 812 | 1898 | 42.78 | 26 | 3.30 to –1.46 |
| 3 | Pylorus | 743 | 1867 | 39.8 | 22 | 3.52 to –1.92 |
| 4 | Pylorus | 790 | 1800 | 43.89 | 20 | 2.98 to –1.50 |
| 5 | Pylorus | 785 | 1788 | 43.9 | 35 | 2.96 to –2.06 |
| 6 | Pylorus | 805 | 1894 | 42.5 | 29 | 3.40 to –1.56 |
| 7 | Pylorus | 760 | 1754 | 43.33 | 22 | 2.60 to –1.42 |
| 8 | Pylorus | 826 | 1920 | 43.02 | 36 | 3.56 to –1.62 |
| **Total** | Pylorus | 6343 | 14822 | 342.46 | 211 | 25.92 to–13.06 |
| **Mean** | Pylorus | 793 | 1853 | 42.81 | 26 | 3.23 to –1.63 |
| **SEM** | Pylorus | 10.43 | 22.17 | 0.46 | 2.24 | 0.13 to –0.08 |

**Abbreviations:**
**DMNV** = Dorsal motor nucleus of the vagus nerve
**nA** = Nucleus ambiguus
**+** = Rostral to the obex
**-** = Caudal to the obex
**mm** = Millimeters
**SEM** = Standard error of the mean

with the pattern of distribution of labeled neurons in the DMNV.

In the four ferrets in which only the pyloric canal was injected, HRP labeled neurons were seen in the DMNV as well as in the nA, while sections taken from the four ferrets which had injections into the pyloric antrum had HRP labeled neurons in the DMNV only.

On examination of the sections taken from the six control ferrets, none of the sections revealed labeled neurons in any of the brainstem nuclei, indicating that the HRP labeling observed in the experimental ferrets were due to HRP injections into the pylorus. The results of the control ferrets also indicate that the vagus nerve is responsible for the neuronal transport of the tracer since vagotomized ferrets did not reveal HRP labeled neurons in the brainstem.

## DISCUSSION

Most of the earlier studies on the innervation of the pylorus had focussed attention on the activities at the peripheral end of the vagus nerve. These activities have been alluded to in the introductory section of this article. The results of some of these earlier studies have led to the knowledge that the pylorus is innervated mainly by the pyloric branches of the hepatoduodenal nerve of Latarjet, the anastomosing branches of the vagus nerve [6,34].

The results of our study, which focuses attention on the central origins of vagal fibres innervating the pylorus, indicate that in the ferret, the pylorus derives its vagal innervation principally from the medial aspect of the DMNV.

The localization of pyloric vagal neurons in the medial aspect of the DMNV in the present study is in complete agreement with an earlier report by Takayama et al [26] in the rat and [38] in the cat. Our results are however only in partial agreement with the report of [24,25] who, following pyloric injection with HRP in the guinea pig found labeled neurons distributed along the entire rostrocaudal extent of the DMNV with no obvious preferred orientation.

The pattern of distribution of labeled neurons in the DMNV in the present study is also in partial agreement with that of Bagaev et al. [27] who localized pyloric neurons in the dorsomedial aspect of the DMNV in the cat, and with that of [39] who demonstrated fairly even distribution of labeled neurons throughout the mediolateral aspects of the DMNV following pyloric antrum injection with HRP in the cat. Since the HRP technique was used by Elfvin and Lindh, [24], Bagaev et al. [27] and Norman et al. [39] as in the present study, the differences in the findings may be due to genuine species difference in the guinea pig, cat and the ferret.

The localization of pyloric vagal neurons in the DMNV in the present study is also consistent with the report of Kressel et al. [28] (1994) that demonstrated pyloric vagal projections from the DMNV by anterograde tracing technique in the rat.

The high percentage of DMNV neurons shown to project to the pylorus in our study (see Table 1) could not be due to labeling of “fibres of passage” since the regions of the pylorus believed to contain these type of fibres [40] were avoided in our injections. What is more likely to be responsible for the disproportionally high percentage of labeled neurons is the phenomenon of “abdominal vagal collateralization”. This phenomenon had been reported in an earlier study by Lizuka and Sugita in the Japenese quail [41], Furthermore, a recent study by one of the authors of this article along with other collaborators has demonstrated collateralization of the abdominal vagal fibres using the fluorescent dye double labeling technique [42].

The results of the present study also showed that the pylorus receives vagal fibres from the nA that was sporadic and sparse. This is a highly significant finding in the present study since to the best of our knowledge, localization of pyloric neurons in the nA has not been reported in any previous study. The result of our study in which we demonstrated projections from the nA to the pyloric canal is consistent with that of Norman and his coworkers [36] who failed to label neurons in the nA following antral injections. The segregation of projections to the antrum and the pyloric canal may be a reflection of the differences in the organization of the musculature of the pyloric canal and the antrum as reported by Cai and his coworker in the guinea pig [43]. The observed segregation in our study may also be on account of the complexities noted in the innervation of the pyloric sphincter compared with the adjoining parts of the sphincter [43].

In an earlier study in the ferret [44], the lesser curvature was reported to contain fibres from the nA and DMNV. This is one of the areas of the stomach, which contains “fibres of passage”. Since the continuation of the lesser curvature to the upper border of the pylorus was not injected in our study, these “fibres of passage” could not

have been responsible for any of the labeling observed in our study. It may well be that the projection observed from the nA to the pyloric canal in the present study is the result of collateralization of vagal fibres en-route to other parts of the gut possibly the liver which receives projections from the nucleus nA. This is consistent with the observation made in the double-labeling fluorescent dye study of Odekunle and his co- workers [42] and the report on vagal innervation of the liver in the ferret [44] Furthermore, since the musculature of the pyloric canal consists of two components, one for closing and the other for opening of the sphincter [21,46-48]. this pattern of organization may be partly responsible for its dual vagal supply from the DMNV and the nA.

Yamamoto et al [23] had reported the origin of pyloric vagal fibres from the medial solitary nucleus in the cat, a carnivore. We found no evidence of such localization in the present study in the ferret, which is also a carnivore. Again, the difference between the two studies in which the same HRP techniques was used might be attributable to genuine species difference.

In conclusion, we have demonstrated for the first time that the ferret derives its vagal pyloric innervation, principally from the DMNV and partly from the nA. We have also shown qualitatively that the projection from the nA to the pylorus is restricted to the pyloric canal in the ferret. Our study has thus complemented available data on the ferret that would facilitate comparison of the ferret with other experimental animal species. Furthermore, the results of our study have not only provided additional information to justify the increasing popularity of the ferret as a suitable animal model in the field of gastrointestinal studies, but have also provided information on what might be the anatomical basis for the already documented functions of the two parts of the pylorus.

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