Brainstem Origin of Duodenal Vagal Preganglionic Parasympathetic Neurons

A WGA-HRP Study in the Ferret (*Mustela Putorius Furo)*, a Human Model

A Odekunle, TI Chinnah

ABSTRACT

The projections of vagal brainstem neurons to the duodenal segment of the gastrointestinal tract were studied in the ferret using the WGA-HRP neurohistochemical technique. Fourteen adult ferrets with weights ranging from 800 gm to 1500 gm were used for the study. The muscular wall of the duodenum of six ferrets was injected with 0.1ml of 5% WGA-HRP in 0.5M sodium chloride. The eight remaining ferrets were used as controls. Two of these had injections of 0.1 ml normal saline into the muscular wall of the duodenum. The second set of two ferrets was injected with 0.1 ml of 5% WGA-HRP in buffer after bilateral truncal vagotomy. The third set of two ferrets received intraperitoneal injection of 0.1 ml of 5% WGA-HRP while, in the last set, the tracer was injected into the hepatic portal vein. Following the injections, the ferrets were allowed to survive for 48-72 hours after which each ferret was perfused transcardially first with normal saline followed by a fixative containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 at room temperature and finally with 10% buffered sucrose at 4ºC. Transverse serial frozen sections of the brainstem were then taken and processed for WGA-HRP neurohistochemistry and were analyzed under light and dark-field illuminations. The analyses of the sections taken from the six ferrets injected with WGA-HRP revealed neurons labelled with the tracer in the dorsal motor nucleus of the vagus nerve (DMNV). Sections taken from the control ferrets did not reveal any WGA-HRP labelled neurons in the brainstem.

Origen Troncoencefálico de las Neuromas Duodenales Vagales Parasimpáticas Pregangliónicas

Un Estudio de WGA-HRP en el Hurón (*Mustela Putorius Furo*), Modelo Humano

A Odekunle, TI Chinnah

RESUMEN

Las proyecciones de las neuronas troncoencefálicas vagales hacia el segmento duodenal del tracto gastrointestinal, fueron estudiadas en el hurón, usando la técnica neurohistoquímica. Catorce hurones adultos con pesos que iban de 800 gm a 1500 gm fueron usados para el estudio. La pared muscular del duodeno de seis hurones se inyectó con 0.1ml de WGA-HRP al 5% en 0.5M cloruro de sodio. Los ocho hurones restantes se usaron como grupo de control. Dos de ellos recibieron inyecciones de 0.1 ml de salina normal en la pared muscular del duodeno. El segundo conjunto de dos hurones fue inyectado con 0.1 ml de WGA-HRP al 5% en buffer después de una vagotomía troncal bilateral. El tercer conjunto de dos hurones recibió una inyección intraperitoneal de 0.1 ml de WGA-HRP al 5% mientras que en el último conjunto, el trazador fue inyectado en la vena porta hepática. Después de las inyecciones, a los hurones se les permitió sobrevivir durante 48-72 horas, tras de lo cual cada hurón fue prefundido primeramente de forma transcardíaca con solución salina normal, seguida por un fijador que contiene paraformaldehído al 1% y glutaraldehído 1.25% en buffer fosfato 0.1M, pH 7.4 a temperatura ambiente y finalmente con sacarosa al 10% en buffer a 4ºC. Entonces se tomaron secciones transversales del encéfalo, seriadas y congeladas, se procesaron para determinar su contenido de WGA-HRP neurohisoquímico, y se aalizaron bajo luz así como bajo iluminaciones de campo oscuro. Los análisis de

From: Anatomy Unit, Department of Basic Health Sciences, Faculty of Medical Sciences, The University of the West Indies, St Augustine, Trinidad and Tobago, West Indies and Department of Biomedical Sciences, University of Sheffield, Sheffield, S10 2TN, England, United Kingdom.

Correspondence: Dr A Odekunle, Faculty of Medical Sciences, Anatomy and Cell Biology, The University of the West Indies, St Augustine, West Indies. Fax: (868)-662-9148, e-mail: odekunle@tstt.net.tt.

las secciones tomadas de los seis hurones inyectados con WGA-HRP revelaron neuronas marcadas con el trazador en el núcleo motor dorsal del nervio vago (DMNV). Las secciones tomadas de los hurones del grupo de control no revelaron ninguna neurona marcada con WGA-HRP en el encéfalo.

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INTRODUCTION

Although the duodenum constitutes a very short segment of the gastrointestinal tract, it plays a vital role in the complex functions of the gastrointestinal system. As the first segment of the small intestine, it receives partially digested material (chime) from the stomach and provides the appropriate medium for continuation of digestion in the small intestine. In addition to receiving bile from the liver, digestive juice and enzymes from the pancreas, the duodenum is also actively involved in the regulation of gastric motility and secretions (1-5). While various studies have indicated that duodenal functions are vagally mediated (6, 7), stimulation of duodenal mechanical and chemical receptors has also been shown to increase vagal afferent firing rate (8-12). In spite of the abundance of literature on vagally mediated duodenal functions, there are very few studies on the precise origin of the vagal fibres implicated in duodenal control (13-17). Furthermore, a review of the literature indicates that a wide range of animal species have been used for duodenal investigations (18-21). Recent studies of gastric physiology (22-24) and gastrointestinal anatomy (25-32) have revealed close similarities between the ferret and the human in respect of these features. The result of these revelations is the increasing popularity of the ferret as a suitable human model in laboratory investigations. In spite of the aforementioned revelations on the ferret and its increasing popularity in laboratory investigations, we have no information on the origin of the vagal fibres innervating the duodenum in the ferret.

The present study was thus motivated by the need to provide the anatomical substrates for reported duodenal activities and to complement already available data on the ferret. The results of this study would also facilitate comparison of the ferret with other species currently used as human models in laboratory investigations.

MATERIALS AND METHODS

A total of 14 male and female adult ferrets, weight range between 800 gm and 1500 gm 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 allowed 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 duodenum, each ferret was anaesthetized with an intraperitoneal injection of sodium pentobarbitone (Sagatal, May and Baker, Dabenham) in a dose of 60 mg/kg body weight. A midline laparotomy was then performed and the duodenum along with the adjoining parts of the stomach and the jejunum delivered to the anterior abdominal wall.

With the aid of a 100 microlitre Hamilton syringe and needle, 0.1 ml of 5% Wheat Germ Agglutinin-Horse Radish Peroxidase (WGA-HRP), (Sigma brand) in 0.5 M saline was injected by multiple penetrations into the muscular coat of the duodenum, from the pyloroduodenal junction to the duodenojejunal junction (Fig. 1). Six ferrets were injected in this manner.

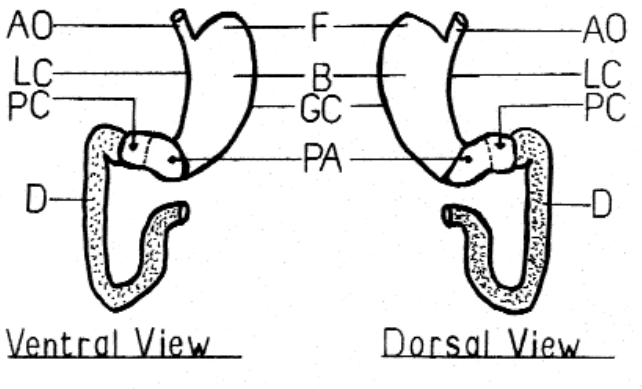

Fig. 1: Schematic representation of the upper part of the subdiaphragmatic part of the gastrointestinal tract, demonstrating the injection sites (dotted areas) of WGA-HRP.
AO Abdominal oesophagus
F Fundus of the stomach
B Body of the stomach
LC Lesser curvature of the stomach
GC Greater curvature of the stomach
PC Pyloric canal
PA Pyloric antrum
D Duodenum (Injection site)

The remaining eight ferrets, which were used as controls, were divided into four sets. The first set of two ferrets was injected in a similar manner as the experimental ferrets with normal saline. The second set of two ferrets was also injected with 0.1 ml of the tracer in a similar manner as the experimental ferret after bilateral truncal vagotomy. The third set of two ferrets was injected intraperitoneally (without performing laparotomy) with the same quantity and percentage of the tracer as the experimental ferrets. In the last set of ferrets, the same quantity of WGA-HRP as in the experimental cases was injected into the hepatic portal vein to determine whether there would be haematogenous transport of the tracer.

In each muscular injection, the needle was left in place at the injection site for about two minutes after injection in order to avoid leakage of the tracer into the peritoneal cavity. After WGA-HRP injection in each experiment, the duodenum was returned to the peritoneal cavity, the laparotomy incision was closed in layers and the animals kept for a survival period of between 48 to 72 hours. At the end of the 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 (pH 7.4) at room temperature and finally with 10% buffered sucrose at 4ºC as recommended by Mesulam (33,34).

The brainstems were then removed, sectioned serially at 20µ thickness with a freezing microtome and processed for WGA-HRP neurohistochemistry using the tetramethyl benzedene method as recommended by Mesulam (33,34).

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/WGA-HRP were seen in their cytoplasm, were regarded as WGA-HRP labelled cells while cells with distinct margin, but not containing any reaction granules, were regarded as unlabelled cells. Both labelled and unlabelled dorsal motor nucleus of the vagal nerve (DMNV) neurons were counted from every second section with the aid of a cell-counting chamber. This was used in calculating the percentage of labelled neurons in each experimental animal.

RESULTS

Injection of HRP-WGA into the wall of the duodenum resulted in the labelling of a substantial number of neurons in the DMNV. Labelled neurons were seen bilaterally and throughout the mediolateral axis of the nucleus (Figs 2,3).

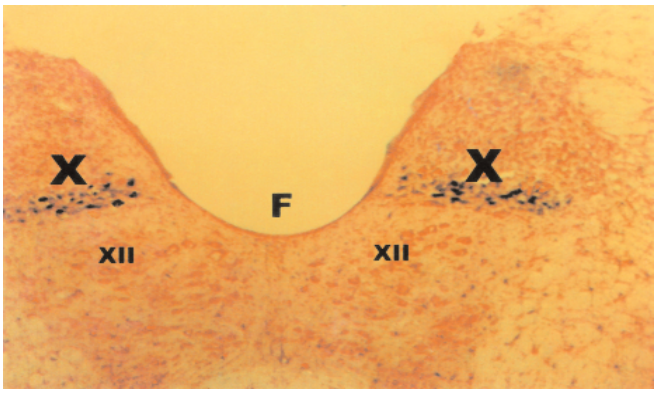

Fig. 2: Photomicrograph of a transverse section through the open part of the medulla oblongata, immediately above the obex. The section demonstrates the presence of WGA-HRP labelled neurons in the left and right DMNV following injections of the tracer into the duodenal wall.
X Dorsal motor nucleus of the vagus nerve,
XII Hypoglossal nucleus and **F** Fourth ventricle

Magnification = (X200)

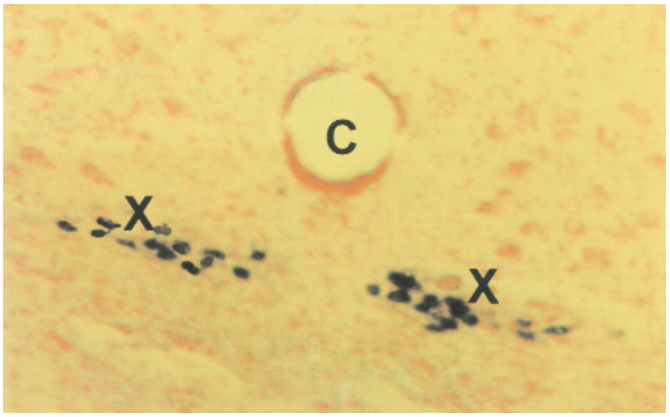

Fig. 3: Photomicrograph of a transverse section through the closed part of the medulla oblongata, caudal to the obex demonstrating WGA-HRP labelled neurons in the DMNV following duodenal injection with the tracer. Note the bilateral labelling of the DMNV and the decrease in number of labelled cells compared with (Fig. 2).
C Central canal, closed part of the medulla oblongata;
X Dorsal motor nucleus of the vagus nerve

Magnification = (X400)

The mean rostrocaudal extent of labelled neurons was from 2.13 mm rostral to the obex to 1.19 mm caudal to the obex (Table).

In the rostrocaudal axis, the highest concentration of labelled neurons were seen in the region of the obex, while in the mediolateral axis, labelled neurons were evenly distributed within the DMNV (Figs 2,3). Furthermore, 27% of the total neurons counted in the DMNV in sampled sections were labelled (Table). No other brainstem nuclei were labelled in the experimental animals.

Concerning the control animals, labelled neurons were not seen in any nuclei in the brainstem in any of the four sets, indicating that there was neither haematogenous transport in the experimental animals, nor transport of the tracer from its deposit in the peritoneal cavity. The failure to label any neuron in the set that had vagotomy also indicates that the tracer labelling observed in the DMNV in the experimental animals was due to neuronal transport via intact vagal fibres innervating the duodenum.

DISCUSSION

The localization of neurons innervating the duodenum in the DMNV in our study confirms the findings of earlier investigators who used the anterograde tracing and electrophysiological techniques to demonstrate innervation of the duodenum by the DMNV (13-16,35,36). In our study, we also observed uniform distribution of duodenal neurons along the mediolateral axis of the DMNV. This finding is also in agreement with those of Kirchgessner (13), Berthoud *et al* (16), and Zhang *et al* (18), all of whom used the retrograde technique as in our study. The similarity in these studies is

Table: Table of statistics of cell count, showing the total number of cells counted in the DMNV in sampled sections in six experimental ferrets and the percentage of labelled cells to total cell count, following multiple injections of WGA-HRP into the duodenal wall. The rostrocaudal extent of labelling of the DMNV is also shown.

Experiment number	Injection site	Number of WGA-HRP labelled cells counted in DMNV	Total number of cells counted in the DMNV	Percentage of labelled to total cells counted in the DMNV	Number of labelled cells counted in the nA	Rostrocaudal extent of labelling of the DMNV
1	Duodenum	478	1814	26.35%	nil	+2.00 mm to -0.9 mm
2	”	510	1810	28.18%	nil	+2.22 mm to -1.9 mm
3	”	498	1792	27.8%	nil	+1.96 mm to -0.74 mm
4	”	470	1798	26.14%	nil	+2.24 mm to -1.8 mm
5	”	513	1805	28.42%	nil	+2.1 mm to -1.08 mm
6	”	510	1802	28.30%	nil	+2.24 mm to -0.74 mm
Total		**2979**	**10821**	**165.19%**		**+ 12.76 mm to -7.16 mm**
Mean		**496**	**1803**	**27.53%**		**+ 2.13 mm to -1.19 mm**
Sem		**±7.49**	**±3.26**	**±0.42**		**± 0.05 mm to ± 0.21 mm**

DMNV dorsal motor nucleus of the vagus nerve, nA nucleus anbiguus, + rostral to the obex - caudal to the obex, ± plus or minus symbol, mm millimetre and Sem standard error of the mean

quite interesting in that whereas the latter investigators used the rat, a herbivore, we used the ferret, a carnivore.

The DMNV is often subdivided into lateral and medial portions along its mediolateral axis. Our observation of uniform distribution of duodenal neurons along the mediolateral axis is suggestive of the duodenum receiving innervation from the lateral and medial portions of the DMNV. Our report is quite interesting and unique as compared with the other segments of the gastrointestinal tract, which have been reported to receive innervation from either the medial or the lateral portions of the DMNV and not from both portions (16,19,37,38). Our finding, with respect to the pattern of distribution of duodenal neurons within the DMNV, is also suggestive of the absence of viscerotopic localization of duodenal neurons within the DMNV and hence the entire duodenal functions could be regulated from either the medial or the lateral aspect of the DMNV. Furthermore, this pattern of innervation may provide the anatomic substrate for the observed integration of duodenal motor activity with that of the distal intestine (21) and integration of duodenal and gastric motor activities (39-42). It is worth investigating the possibility that one aspect of the DMNV innervating the duodenum may be implicated in integrating duodenal and distal gastrointestinal segments while the other aspect might be responsible for integration of duodenal and proximal gastrointestinal segments.

As regard the 27% of the DMNV neurons observed to innervate the duodenum in the present study, this appears to be quite high, particularly against the background of a previous study in the ferret in which 76% of DMNV neurons was reported to innervate the stomach (30) which receives its vagal fibres from the same abdominal vagal trunks as does the duodenum. It is most likely that the 27% observed here are not exclusive to the duodenum since earlier studies have also shown that the gastric, celiac and the hepatic branches of the abdominal vagal trunk contain fibres innervating the duodenum (16,17). It is therefore logical to assume that some of the neurons observed to be innervating the duodenum in our study may also be involved in the innervation of other abdominal viscera through the process of axonal collateralization. This assumption is consistent with the findings reported by Odekunle *et al*. (43), who investigated the

phenomenon of collateralization of axons of DMNV neurons in the rat using the double-labelling fluorescent dye technique.

In conclusion, our study has provided the first documented evidence on the precise origin of the preganglionic parasympathetic vagal fibres innervating the duodenum of the ferret. We have also compared the results of our finding with reports of similar studies in other species. The functional implications of the pattern of vagal innervation of the duodenum in our study were also addressed.

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