The Brainstem Localization of Gastric Preganglionic Parasympathetic Neurons in the Agouti (*Dasyprocta leporina*)

A Odekunle, CM Phillips

ABSTRACT

This study was designed to determine qualitatively, the source of gastric vagal nerve fibres in the Agouti. A total of 18 male and female adult agoutis were used for the present investigation. Following anaesthesia, laparotomy was performed and the stomach exteriorized. Multiple intramuscular injections of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) were then made into different areas of the stomach in the experimental animals. The control animals were divided into four groups of two animals each. The first group had intraperitoneal injection of the tracer, the second had intramuscular injection of normal saline, the third group had injection of tracer into the hepatic portal vein and the last group had injection of the tracer into the gastric walls followed immediately by bilateral vagotomy. Following a survival period of five to seven days, the animals were sacrificed by transcardial perfusion, first with normal saline followed by fixative and finally with 20% buffered sucrose. Following perfusion, the brainstem was extracted from the brain, immersed in 20% buffered sucrose and kept refrigerated overnight for cryoprotection. The brainstems were subsequently sectioned serially, processed for WGA-HRP neurohistochemistry and then analysed under light and dark-field illuminations. The analysis of the sections taken from the experimental animals revealed bilateral presence of WGA-HRP labelled neurons in the dorsal motor nucleus of the vagus nerve (DMNV) and the nucleus ambiguus (nA) of the medulla oblongata. No labelled neurons were seen in any of the sections taken from the control animals. The implications of the findings are discussed.

Keywords: Agouti, gastric neurons, neurohistochemistry, wheat germ agglutinin-horseradish peroxidase (WGA-HRP)

Localización del Tronco Encefálico de las Neuronas Parasimpáticas Preganglionares Gástricas en el Agutí (*Dasyprocta leporina*)

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RESUMEN

Este estudio fue diseñado para determinar cualitativamente el origen de las fibras gástricas del nervio vago en el agutí. Un total de 18 agutíes adultos masculinos y femeninos fueron utilizados para la presente investigación. Después de la anestesia, se realizó una laparotomía y se sacó el estómago al exterior. Luego se hicieron múltiples inyecciones intramusculares de aglutinina de germen de trigo con peroxidasa de rábano (WGA-HRP) en diferentes áreas del estómago de los animales experimentales. Los animales del control fueron divididos en cuatro grupos de dos animales cada uno. Al primer grupo se le puso una inyección intraperitoneal del marcador; al segundo se le administró una inyección intramuscular de solución salina normal; al tercer grupo se le inyectó el marcador en la vena porta hepática; y al último grupo se le puso la inyección del marcador en las paredes gástricas, seguida inmediatamente por una vagotomía bilateral. Tras un periodo de supervivencia de cinco a siete días, los animales fueron sacrificados por perfusión transcardíaca, primero con solución salina normal, seguida de fijador, y finalmente con sacarosa tamponada al 20%. Después de la perfusión, el tronco encefálico fue extraído del cerebro, inmerso en sacarosa tamponada al 20%, y mantenido en refrigeración durante la noche para su crioprotección. Los tronos encefálicos fueron luego

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seccionados en serie, procesados para para el análisis neuro-histoquímico mediante aglutinina de germen de trigo con peroxidasa de rábano, y analizados entonces bajo iluminaciones de campo de luz y campo oscuro. El análisis de las secciones tomadas de animales experimentales reveló la presencia bilateral de neuronas etiquetadas WGA-HRP en el núcleo motor dorsal del nervio vago (DMNV) y en el núcleo ambiguo (nA) de la médula oblonga. No se observaron neuronas etiquetadas en ninguna de las secciones tomadas de los animales de control. Se discuten las implicaciones de los hallazgos.

Palabras claves: Agutí, neuronas gástricas, neuro-histoquímica, aglutinina de germen de trigo-peroxidasa de rábano (WGA-HRP)

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INTRODUCTION

The enormous advances of science and medicine over the past century would have been impossible were it not for the extensive use of animals in research (1). The value of experimental animals in medical research cannot be overemphasized as their usage has made acquiring conclusive scientific data faster and with fewer ethical constraints.

Of all the known mammalian species, approximately half belong to the order Rodentia (2). Animals in the order Rodentia that are presently used as experimental animals include the guinea pigs, hamsters, rats and mice. The rat and mouse constitute well over 80% of the animals utilized in medical and other scientific inquiries (3, 4). In 2007, statistics released by the Home Office in the United Kingdom alone revealed that 83% of 3.2 million scientific animal procedures were done using rodents (5).

The rationale for selecting the rodent species for laboratory biomedical research includes:

* They have anatomical, metabolic, physiological and pathological properties that closely parallel those of humans
* They possess biological (fast reproductive rate) and physical (small size) characteristics that make them more suited for planned studies
* Their internal (food, surgery) and external (temperature, light) environments can be easily manipulated and controlled thus minimizing conflicting variables
* They have been well characterized genetically and are manipulated to produce defined strains
* Rodent species are commonly cited and utilized as animal models in most peer reviewed literatures (6) and their baseline anatomy, physiology and biochemistry are well characterized

Furthermore, it has been predicted that there would be an increase in the number of rodents utilized in scientific studies, as scientist would require *in vivo* systems to validate new discoveries in molecular biology and genetic research (7, 8). It has also been observed that while rats and mice are quite suitable for some investigations, they appear to be too small for other investigations.

In an effort to facilitate its domestication and adoption as an experimental animal, the agouti, a neotropical rodent of the family Dasyproctidae (4), local to Trinidad and Tobago and some other Caribbean islands (9), was chosen as the experimental animal for the present study. The agouti has been cited as a potential animal that could be suitable as a human model for four main reasons (5):

* Its larger size (3.5 to 5 kg) compared with the rat (10−12)
* Longevity of life (18 to 20 years life span (12))
* Easy maintenance in captivity (10−12)
* Its resistance to zoonotic diseases (12)

To date, several anatomical and physiological investigations have been carried out on the agouti (12−25). While these investigations were quite diverse, the goal is to add to the growing database of the agouti. The present study is an immediate follow-up of our earlier study of the organization of brainstem nuclei in the agouti (26).

SUBJECTS AND METHODS

A total of 18 adult male and female agoutis, weight range between 2.5 and 5 kilograms were utilized for the present study.

Following physical examination to ensure they were healthy enough to survive the experiments, they were each kept in separate cages in a controlled environment. Each cage was fitted with separate water and food receptacles to which they had free access. Food and water were often withheld from the animals about 12 hours before surgeries.

For the experiments, 5 mg of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) was diluted with 100 μL of 0.9% saline solution and used for each experimental animal as well as control animals that were injected with the tracer. With the aid of a Hamilton syringe, intramuscular injections of WGA-HRP were made by multiple penetrations into the stomach wall. The injections were made within the muscular coat of the stomach under the guidance of a dissecting microscope and care taken not to inject into the gastric lumen.

The following areas of the stomach were injected: the anterior and posterior walls, the lesser and greater curvatures and the cardia. The needle was slowly withdrawn after each penetration and the injection site pressed down with sterile, dry gauze for approximately one minute to prevent leakage of the tracer.

The control experimental animals were divided into four groups. The first group had intraperitoneal injection of the tracer through the anterior abdominal wall without laparotomy. The second had intramuscular injection of normal saline into the gastric walls, the third group had injection of the tracer into the hepatic portal vein and the last group had injection of the tracer into the gastric walls followed immediately by bilateral vagotomy. The quantity of tracer injected in all controls was the same as for the experimental animals.

After the injections, the stomach was returned to its anatomical position and the abdominal incision was closed in layers.

The animals were subsequently allowed a survival period of between five and seven days following which they were sacrificed by transcardial perfusion, first with 500 mls of normal saline, followed by 1 litre of fixative containing a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde, pH 7.4 at room temperature and finally with 1 litre of 20% buffered sucrose, pH 7.4 at 4 °C.

Following perfusion, craniotomy was performed and the brain, together with adjoining parts of the spinal cord, was detached. The brainstem was then extracted from the brain and immersed in 20% buffered sucrose, pH 7.4 and kept refrigerated overnight for cryoprotection. Following cryoprotection, the brainstem was sectioned serially at 50 µm thickness with the cryostat and the sections processed for WGA-HRP neurohistochemistry as recommended by Mesulam (27, 28). The sections were then stained with neutral red and then analysed under light and dark-field illuminations with the Nikon microscope coupled to a Nikon camera and Dell computer.

RESULTS

After the tetramethyl benzidine (TMB) neurohistochemical procedure and counterstaining with neutral red, labelled cells were readily identifiable as they were densely stained with a blue-black precipitate contrasting with a pink background. In some cells, the protoplasmic extensions were also visible.

Inoculations of WGA-HRP into all the areas of the stomach resulted in bilateral labelling of neurons in the dorsal motor nucleus of the vagus nerve (DMNV). The labelled neurons were observed along the rostrocaudal extent of the nucleus and were generally located at its dorsomedial aspect (Fig. 1).

The rostrocaudal extent of labelling was from 1.7 mm caudal to 1.8 mm rostral to the obex. It was also observed that the density of labelled neurons was higher in the caudal part of the nucleus up to the level of the obex. Furthermore, sections taken from the anterior and posterior walls showed higher density of labelled neurons compared to those taken from the curvatures and the cardia. The lowest population of labelled neurons was observed in the sections taken from the greater curvature of the stomach. In a few sections taken from the ventral and dorsal gastric walls, some labelled neurons were seen in the region of the central gray matter of the upper two cervical segments of the spinal cord (Fig. 2).

None of the sections taken from the control experiments revealed any labelled neuron in the DMNV.

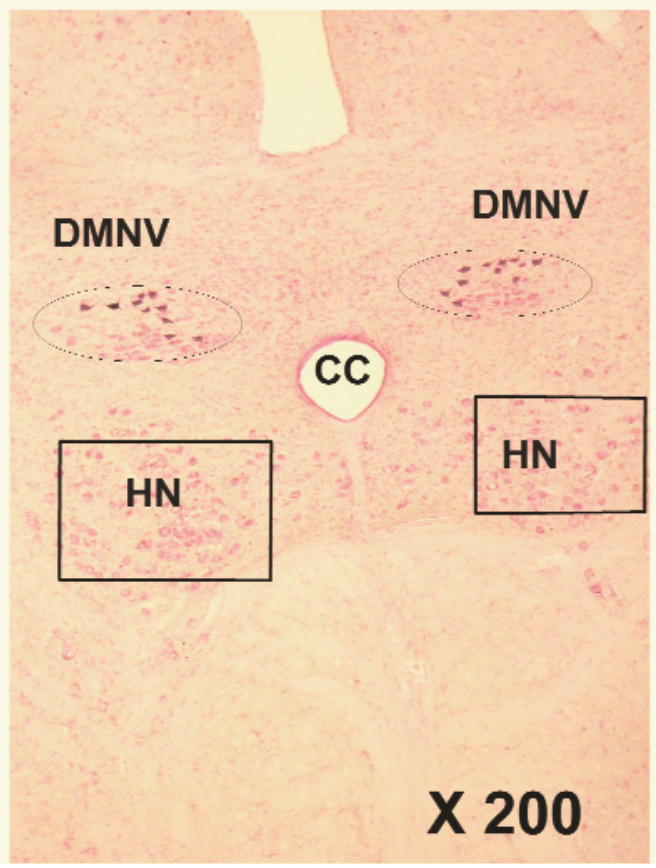

Fig. 1: Transverse section through the closed part of the medulla oblongata showing labelled neurons of the DMNV.

DMNV – dorsal motor nucleus of the vagus nerve; HN – hypoglossal nucleus; CC – central canal, closed part of the medulla oblongata

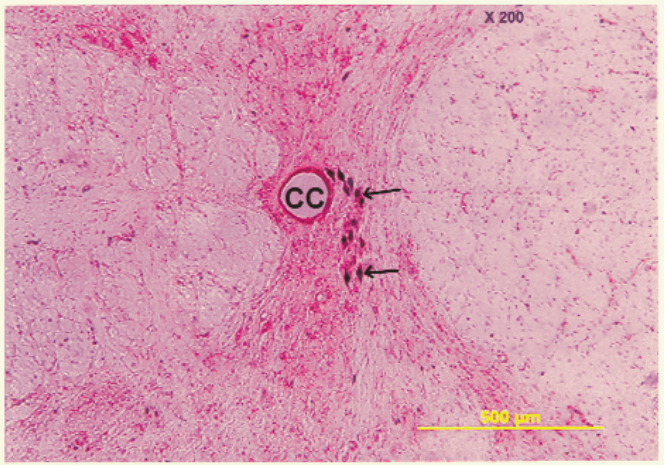

Fig 2: Transverse section through the second segment of the spinal cord showing labelled neurons (arrowed).

CC – central canal of the spinal cord

As regards the nucleus ambiguus (nA), few labelled neurons were seen in the sections taken from the lesser curvature and cardiac injections (Fig. 3). The labelled

Fig 3: Transverse section through the opened part of the medulla oblongata showing labelled neurons (arrowed) in the nucleus ambiguus.

neurons in this nucleus were restricted to the intermediate region of the nucleus. The rostrocaudal extent of labelling was about 0.9 mm from the level of the obex upwards. None of the control experiments demonstrated the presence of labelled neurons in the nA.

Control *vs* experimental group

Although the evidence was qualitatively compelling, there was a need to quantify the number of cells stained. The NIH Image J programme was utilized for this purpose and a one way analysis of variance (ANOVA) test was applied to estimate the significance of the differences between the control and experimental groups. When compared to the control group, the intensity of labelling seen was indeed significantly different [$p < 0.05$] (Figs. 4 and 5, Table).

Table: Two-sample *t*-test and confidence interval for the experimental and control groups

	n	Mean	St Dev	SE Mean
EXP	10	153.61	1.75	0.55
CTR	10	169.31	7.05	2.2

Difference = mu (EXP) - mu (CTR); Estimate for difference: -15.71; 95% CI for difference: (-20.82, -10.59); *t*-test of difference = 0 (*vs* not =): T-value = -6.84; *p*-value = 0.000, DF = 10

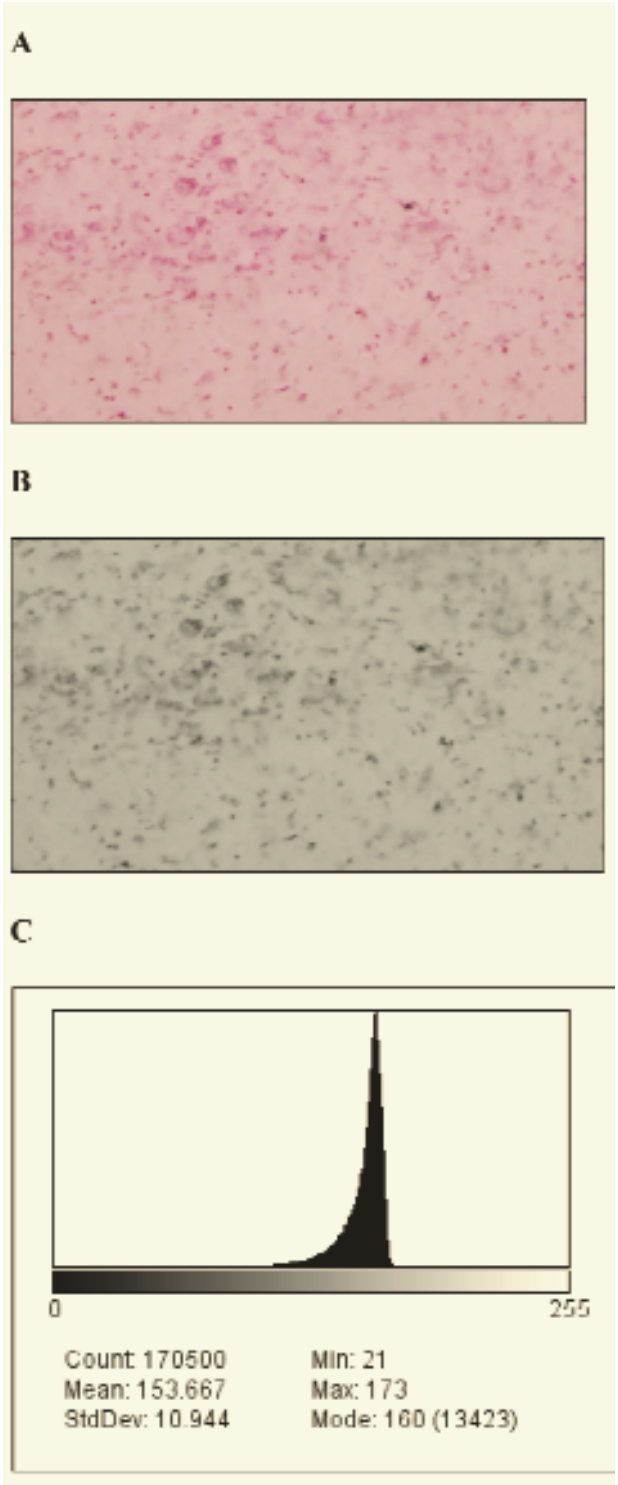

Fig. 4 (A–C): Photomicrographs and histogram of image J output for control group of experiments.

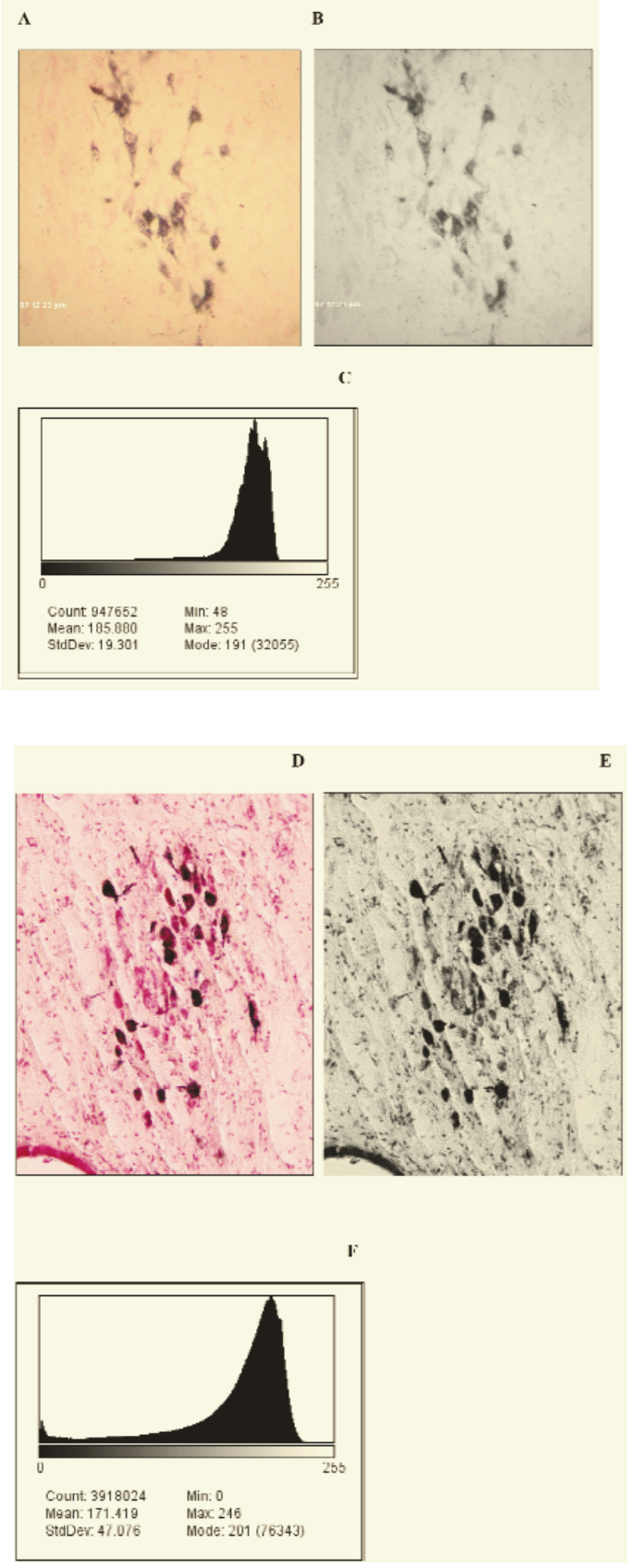

Fig. 5 (A–F): Photomicrographs and histogram of image J output for experimental group of animals.

DISCUSSION

The principal objective of the current neuroanatomical study was to determine the central origin of the preganglionic vagal efferent fibres innervating the stomach in the agouti using WGA-HRP neuronal tracer.

To date, vagal innervation of the stomach by the DMNV has been reported in the rat (29−32), cat (33, 34, 35), sheep (36), guinea pig (37), squirrel monkey (38), house musk shrew (39), ferret (40−43), dog (44) and the rabbit (45).

The nA has also been reported to be implicated in vagal innervation of the stomach in the sheep (36, 46), squirrel monkey (38), cat (34, 47, 48), ferret (49, 50), mouse (51) and rat (32, 52−55).

The major findings in the present study suggest that:

* Efferent fibres projecting to the stomach arise from the dorsomedial aspect of the DMNV
* The nA also projects to certain parts of the stomach

With regard to the DMNV, our findings appear to be consistent with those previously described for the laboratory rat (56), sheep (36), dog (44), rabbit (45), house musk shrew (39) cat (34, 57) and the ferret (41, 42, 49).

While the present study localized vagal neurons in the dorsomedial aspect, the investigators of the ferret localized vagal neurons in the ventromedial aspect of the nucleus. The difference in the localization of vagal neurons in the agouti and ferret could possibly be attributed to species difference, the agouti being a herbivore while the ferret is a carnivore. Furthermore, the findings of the present study are partly in agreement with those of the investigators who traced vagal fibres to the medial aspect of the DMNV in the rat (31, 56, 58). The observation of a high density of labelled neurons in the region of the obex in the present study also mimics the findings of Won *et al* in the house musk shrew (39).

In two cases in the present report, labelled neurons of the DMNV extended into the spinal cord. This phenomenon had been previously demonstrated by Scharoun *et al* who observed labelled neurons in the laminae X regions of the first two cervical segments of the hood rat (59). This occurrence was also recorded in the ferret in which a dorsomedial nucleus was labelled on injection of the stomach. The investigators of the ferret referred to this group of labelled neurons as aberrant neurons of the DMNV.

While earlier investigations have demonstrated the DMNV as the principal site of origin of gastric vagal efferent neurons (33, 42, 60, 61), there have been reports that some species, such as the rat, feline and dog, are innervated dually by the DMNV and the nA (35, 37, 38, 54).

The present report in which the nucleus ambiguus was labelled following injections into the cardia and lesser curvature is in agreement with the latter observation. It is, however, worth noting that the labelling observed in the nA in the present study and perhaps in other similar studies does not necessarily imply that the stomach is innervated by the nA since the cardiac and lesser curvature are regions of high

traffic of fibres, some of which are "fibres of passage" en route other sites of the gastrointestinal tract distal to the stomach (42).

It is most probable that Won *et al* (39) localized neurons in the nA for the same reason of the location of "fibres of passage" in the abdominal oesophagus and the cardiac region of the stomach in the house musk shrew.

The failure to label neurons in all the control experiments in the present report indicates that the neuronal tracer used in this study was transported by the vagus nerve and not through the blood stream.

In conclusion, the present study has localized for the first time ever gastric efferent neurons in the dorsal motor nucleus of the vagus nerve in the agouti. The present study is also of the opinion that the labelled neurons seen in the nA following injections into the lesser curvature and gastric cardia may not be neurons projecting to the stomach but rather traversing those areas of the stomach en route to lower segments of the gastrointestinal tract. The latter fibres are conventionally referred to as "fibres of passage".

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