J. Anat. (1989), **163**, pp. 173–181
With 3 figures
Printed in Great Britain

The innervation of the adrenal gland. III. Vagal innervation*

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(*Accepted* 16 *July* 1988)

INTRODUCTION

It was in the late nineteenth century that good physiological evidence of the functional innervation of the adrenal gland was obtained by direct stimulation of the greater thoracic splanchnic nerve in the dog (Dreyer, 1899). Since that time many workers have confirmed and extended these findings and others have experimentally demonstrated the segmental origin of preganglionic neurons concerned with the innervation of adrenal chromaffin tissue (for review of early literature see Coupland, 1965*a*, *b*) and more recently the perikarya of both pre- and postganglionic neurons innervating the rat adrenal gland have been demonstrated (Kesse, Parker & Coupland, 1988).

Investigations aimed at determining whether or not the adrenal gland is also innervated by the parasympathetic nervous system through vagal fibres have, in general, resulted in negative answers. However, Teitelbaum (1933) in a gross anatomical study in man confirmed and extended previous observations of Kollmann (1860) who had claimed to have traced nerve fibres from the posterior vagus cord in the subdiaphragmatic region to the left adrenal gland.

The work now reported was undertaken using Fast Blue (FB) and horseradish peroxidase (HP) as retrograde tracers of nerve fibres following their injection into the left adrenal gland. The animals used were in some instances also used for studies on the sympathetic efferent and sensory innervation of the adrenal gland, and control experiments were performed to ensure that uptake and transport were not a consequence of leakage into the peritoneal cavity or vascular injection.

MATERIALS AND METHODS

Animals used were Wistar rats of 250 ± 10 g weight, obtained from the University of Nottingham Animal Breeding Unit, Sutton Bonnington, and Dunkin Hartley guinea-pigs aged 1–2 months (380–420 g) supplied by the Nottingham University Medical School Animal House. Animals of both sexes were included. They were housed in the Medical School Animal Unit under standard laboratory conditions of temperature 21 °C and lighting (12 hours light, 12 hours dark). The animals had free access to food (rats to Diet 41B, Heygates Ltd, Northampton, guinea-pigs to SGIACS, Heygates Ltd, Northampton) and water, which for guinea-pigs contained 1 g of vitamin C/500 ml.

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Prior to injection of tracers, rats were anaesthetised with an intraperitoneal injection of 60 mg/kg body weight of sodium pentobarbitone (May & Baker Ltd, Dagenham, Essex); if supplementary anaesthesia was required this was given as an intramuscular injection of 50 μl of Hypnorm (Janssen Pharmaceuticals, Oxford, England).

Guinea-pigs were anaesthetised by intramuscular injections of first diazepam (2·5 mg/kg; Roche Products Ltd, Welwyn Garden City), followed by 1 ml/kg Hypnorm (fentanyl base 0·2 mg/ml, fluanisone 10 mg/ml).

Anaesthesia sufficient to abolish the stretch reflex in the lower limbs was achieved prior to surgery. The left adrenal gland was exposed by a posterolateral approach and immobilised by applying to its surface a 15 G hypodermic needle attached to a suction pump. Tracer was then injected and subsequently the gland was returned to its original position and the abdominal wall closed with 3/0 catgut and the skin with Michel clips.

In the guinea-pig the left adrenal gland was exposed through an anterior midline sagittal incision and the wound closed with 3/0 catgut and Michel clips.

Prior to tracer injection the adrenal capsules were pierced by the tip of a hypodermic needle to enable the micropipette to enter the gland. Three to five μl of a 2% aqueous suspension of Fast Blue (Dr. Illing Makromolekulare Chemie, Gross-Umstadt, FGR) or 30% solution of horseradish peroxidase (HRP: Sigma type VI, Sigma Chemical Co, St Louis, USA) was injected under pressure into the adrenal gland over a period of 15–20 minutes via a glass micropipette (tip diameter 20 μm) coupled to a Hamilton syringe (Scientific Glass Engineering PTY Ltd, Ringwood, Australia) by means of a piece of nylon tubing. The micropipette was held on a stereotactic frame (David Kopf Instruments, Tujunga CA, USA). After the injection the micropipette was slowly withdrawn over a period of 10 minutes, and the point of entry sealed by electrocautery.

Injected animals were returned to the animal house for 4 days (HRP) or 7 days (FB) after which they were anaesthetised by an intraperitoneal injection of sodium pentobarbitone and then perfused transcardially for 1 minute (rats) or 2 minutes (guinea-pigs) at 120 mmHg pressure with a prewash containing 5% dextran 70 and 1% procaine hydrochloride at body temperature, followed by fixative. Fast Blue-injected animals were fixed with 10% depolymerised paraformaldehyde in 0·1 M phosphate buffer, pH 7·3. HRP-injected animals were fixed with 1·25% glutaraldehyde (Taab Laboratories Equipment Ltd, Reading, Berks) and 1% depolymerised paraformaldehyde in 0·1 M phosphate buffer pH 7·3%. After 20 minutes fixation-perfusion some of the animals were perfused with 10% sucrose (w/v) in 0·1 M phosphate buffer for a further 10 minutes prior to removal of the cervical vagus nerves, including sensory ganglia and the brainstem from the level of the foramen magnum to the midbrain; in other animals these tissues were dissected out immediately after fixation, immersed in the same fixative for 90 minutes and then left overnight in 10% sucrose in 0·1 M phosphate buffer.

The segments of the upper cervical portions of vagus nerves from rats, which included the sensory ganglia, were embedded in fresh ox liver; guinea-pig vagi were embedded in plastic JB-4 (Polysciences Ltd, Moulton Park, Northampton). These were then cut, respectively, on a cryostat (Bright Instruments) at a thickness of 20 μm or on a Jung microtome at 10 μm.

Frozen sections of rat brainstem were cut in the cryostat in the longitudinal plane (coronal) at 25 μm. The guinea-pig brainstem was cut in the same plane at a thickness of 60 μm using a freezing sledge microtome. Sections were mounted on chrome alum–gelatin-coated slides, air dried and stored in the refrigerator until examined.

Sections prepared from HRP-injected animals were rehydrated and processed for

HRP histochemistry using the tetramethylbenzidine technique of Mesulam (1978), some sections being stained with acetate buffered (pH 4·8) neutral red, and mounted in DPX. They were then examined with ×6·3 air and ×25 and ×64 oil objectives using a Leitz Ortholux microscope. Photographs of labelled cells were taken on Kodak Ektachrome 50 ASA or Ilford Pan F 135 film using a Leitz-Wild Photoautomat MP555.

Sections from FB-injected animals were air dried and mounted in non-fluorescing Fluorolite mounting medium (Raymond Lamb, North Acton, London) and examined with the above objectives using a Leitz Ortholux microscope fitted with a Ploempack fluorescent system and excitation wavelengths of 360 and 390 μm.

When cell counts were made, only labelled cells that contained a nucleus were recorded so as to reduce the possibility of double counting.

Control experiments included the examination of the same tissues from non-injected animals and after the intravenous injection of 3–5 μl FB into the rat tail vein. No labelled neurons were observed in either central or peripheral nervous system.

The site of injection within the adrenal gland was determined by sectioning the gland and processing as for the brainstem. In all instances the tip of the micropipette at the time of injection was within the adrenal medulla in a central or paracentral position and no spread back to the surface of the gland had occurred.

In 4 rats and 10 guinea-pigs the vagus nerve was exposed on the right or left side of the neck prior to injection of the adrenal gland. In equal numbers of animals the left or right vagus was sectioned in the lower cervical region and a segment of nerve about 0·5 cm long excised: the wound was closed with 3/0 catgut and Michel clips. Following recovery of the animals the adrenal glands were injected 4–5 days later and tissues processed as above.

RESULTS

Rat

Sensory neurons

The left adrenal glands of 7 rats were injected with HRP and 10 were injected with FB.

In the rat the sensory neurons of the vagus nerve are difficult to separate into distinct superior and inferior ganglia, and the description of labelled cells relates to both combined. The perikarya of the neurons are found in an elongated swelling extending for some 3 mm below the jugular foramen and commonly referred to as the nodose ganglion.

In all injected animals, either HRP- or FB-labelled neurons were observed in both ipsi- and contralateral vagal sensory (nodose) ganglia. The labelled cells were rounded, polyhedral or fusiform in shape and randomly distributed throughout the ganglion on each side. Rounded cells had a diameter of 19–22 μm. The long axis of fusiform cells (28 μm) measured twice that of the short axis (14 μm). In 7 animals the number of FB-labelled cells (Fig. 1) in the left nodose ganglion was $15{\cdot}4 \pm 2{\cdot}5$ (mean $\pm$S.E.) the range being 4 to 23 cells. In the contralateral (right) ganglion the labelling was $11{\cdot}8 \pm 1{\cdot}9$, range 6 to 22 cells. Hence slightly more ipsi- than contralateral vagal sensory neurons were labelled. In HRP-injected animals reaction product was also seen in the process of ganglion cells (Fig. 1).

In unilaterally vagotomised rats no labelled neurons were observed in the nodose ganglion ipsilateral to the section but they were always observed in numbers

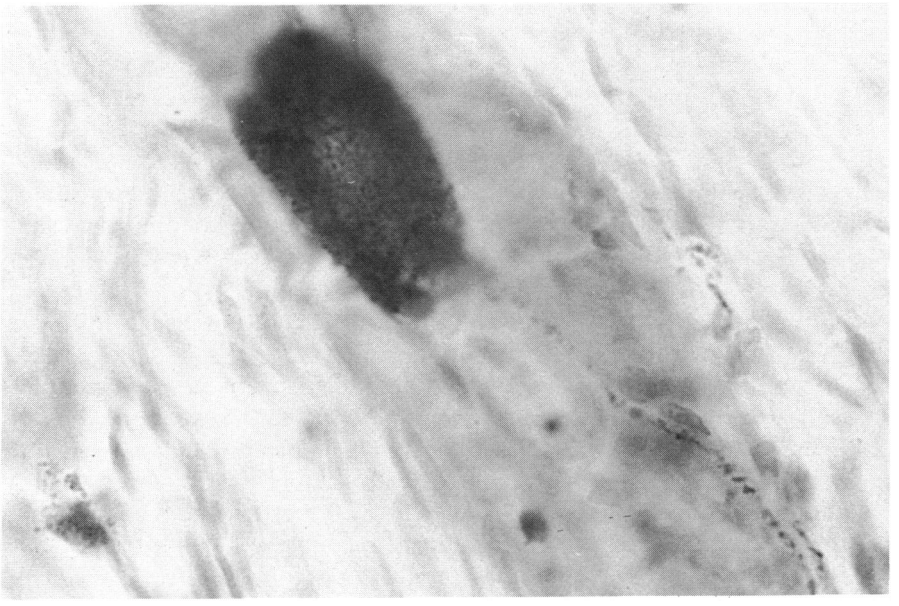

Fig. 1. Neuron perikaryon and process retrogradely labelled with horseradish peroxidase in the nodose ganglion of the rat. ×900.

compatible with those observed in the right or left nodose ganglia of normal animals (non-vagotomised) in the ganglion contralateral to the section.

Motor neurons (dorsal motor nucleus of the vagus)

In 2 of 10 normal animals in which FB had been injected into the left adrenal gland, labelled neuronal perikarya were observed in both ipsi-(left) and contralateral (right) dorsal motor nuclei of the vagus.

In one rat 12 labelled neurons were seen in the left nucleus and 8 in the right. In the other animals in which labelling had occurred the counts were 10 left and 5 right. As compared with the fluorescence in labelled sympatho-adrenal neurons (Kesse *et al.* 1988) the fluorescence in the vagal nuclei was of moderate rather than high intensity but no evidence of labelled nerve fibres that may suggest transynaptic spread to motor neurons were observed.

No other brainstem nuclei were labelled and no labelling was observed after vagotomy.

Guinea-pig

In the guinea-pig both superior and inferior (nodose) ganglia of the vagus nerve can be identified. The superior ganglion, which is spherical, lies in a sheath with the accessory nerve and the inferior ganglion forms an elongated swelling as the Xth and XIth nerves lie side by side. Just distal to the inferior ganglion branches pass medially and caudally towards larynx and thyroid gland.

Sensory neurons

The vagal ganglia were examined in 10 animals in which FB had been injected into the left adrenal gland. Labelled cells were observed, randomly distributed, in both superior and inferior ganglia of right and left vagi; they were oval or polyhedral in shape (Fig. 2) and ranged in size from 20 to 35 μm in the long and 12 to 18 μm in the

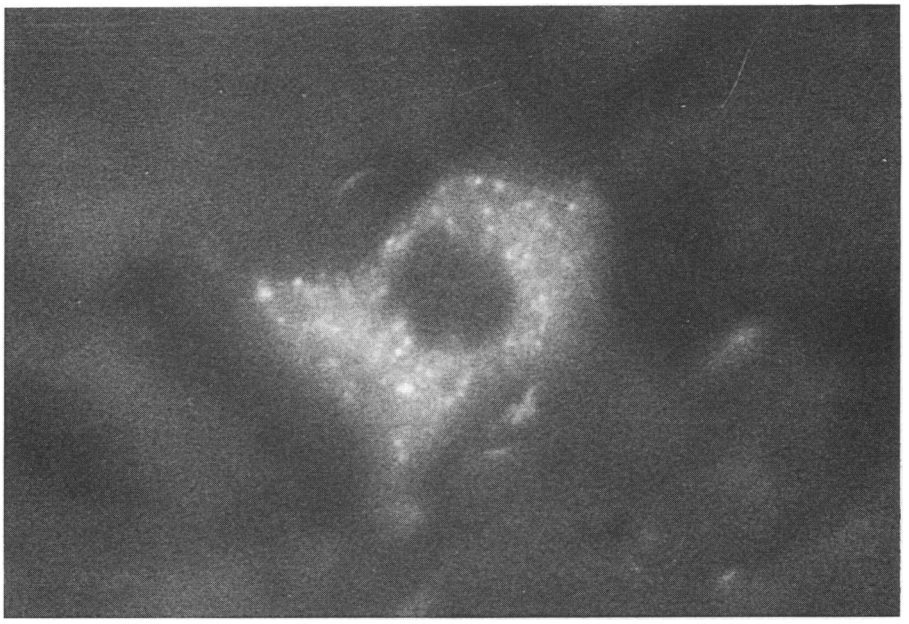

Fig. 2. Retrogradely labelled neuron in the nodose ganglion of the guinea-pig after injection of Fast Blue into the adrenal medulla. × 1250.

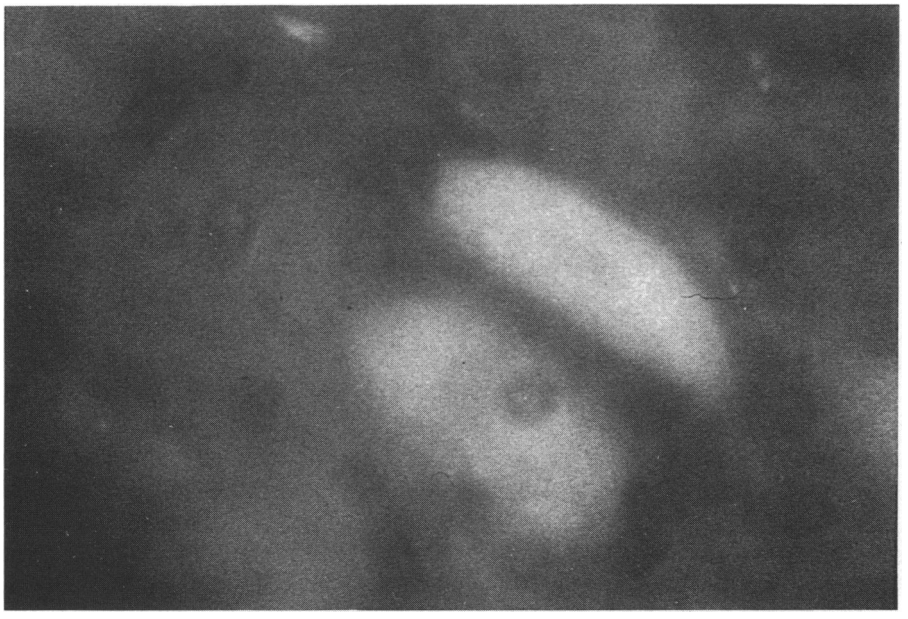

Fig. 3. Two retrogradely labelled neuron perikarya in the dorsal motor nucleus of the vagus after the injection of Fast Blue into the guinea-pig adrenal medulla. × 750.

short axis. In counterstained sections the labelled cells conformed in appearance to the many unlabelled cells of the vagal sensory ganglia.

In 5 animals cell counts were made and these showed $7{\cdot}8 \pm 0{\cdot}8$ (mean $\pm$ S.E.) labelled cells in the superior and $18{\cdot}6 \pm 2$ in the inferior ganglion on the left side and $5{\cdot}6 \pm 1{\cdot}0$ in the superior and $17{\cdot}0 \pm 1{\cdot}1$ in the inferior ganglion on the right side.

Following unilateral vagotomy in which the left vagus was cut, no labelled cells were observed in either right or left vagal sensory ganglia. However, when a right vagotomy was performed labelled cells were seen in both the superior and inferior left vagal ganglia.

Motor neurons (dorsal motor nucleus of the vagus)

Labelled neurons were found in both the right and left dorsal motor nucleus of the vagus in all animals after FB injections into the left adrenal gland. Labelled cells extended more or less in line from the cranial to the caudal limits of the nucleus on each side and varied in shape from oval (Fig. 3) to round or polyhedral and from 30 μm to 18 μm in long and short diameters. In counterstained sections they resembled the adjacent non-labelled neurons. The nuclei had a mean diameter of 7·2 μm.

The number of labelled neurons was $70{\cdot}6 \pm 2{\cdot}9$ (mean $\pm$ S.E.M.) in the left (ipsilateral) dorsal nucleus of the vagus and $52{\cdot}3 \pm 2{\cdot}1$ in the right nucleus.

After left vagotomy no labelled neurons were seen in the brainstem. After right lower cervical vagotomy, neurons within the normal range of sizes (see above) were seen in the dorsal motor nucleus of the vagus on the left side only.

DISCUSSION

The present findings provide unequivocal evidence for a sensory vagal innervation of the adrenal gland in both the rat and the guinea-pig. In the rat a sensory innervation appears to predominate, and the labelled neurons present possess the typical morphological features of primary afferent neurons.

In the guinea-pig the number of labelled neurons with perikarya in the dorsal motor nuclei of the vagi is some 2–3 times as great as those with perikarya in the nodose ganglia. Hence it is apparent that, in the guinea-pig, vagal innervation is predominantly efferent and the left adrenal gland is innervated by bilaterally situated motor and sensory neurons with usually an ipsilateral predominance.

In inserting the micropipette in the present series of experiments an attempt was made to position the tip in the centre of the adrenal medulla. Examination of the adrenal glands of animals confirmed that the tip was always situated within the medulla in a central or paracentral position. Furthermore, the injected FB or HRP was largely confined to the medulla, extending outwards only as far as the inner cortex (zona reticularis) except in the zone adjacent to the track of the micropipette, where it sometimes extended peripherally as far as the glomerulosa/fasciculata junction.

Given the site of injection and mainly medullary localisation of tracer at the time the animals were fixed it is likely that the labelled neurons had endings within the adrenal medulla, though there is a possibility that transient 'flash' vascular labelling of the whole gland may have occurred in the light of the nature and complexity of the adrenal vascular bed (Coupland, 1975; Coupland & Selby, 1976). Clearly the location of both visceral afferent and efferent endings within the adrenal medulla must be determined by antegrade transport studies and these are currently under way in this laboratory.

The variability of results with respect to the labelling of neurons in the dorsal motor nucleus of the vagus in the rat warrants further comment. In two out of ten rats injected, labelling of neurons in the dorsal motor nucleus of the vagus was observed. This could reflect non-specific uptake of label, possibly from uptake of dye following vascular transport, but this is highly unlikely owing to the small amount (3–5 μl) of Fast Blue injected very slowly and the fact that in control experiments in which the dye

was injected intravenously no such labelling was observed in the brainstem or elsewhere in the central or peripheral nervous system. It is of interest to note that very similar results were obtained by Donovan, Wyss & Winternitz (1983) and Gattone, Marfurt & Dallie (1986) following injection of fluorescent dye into the kidney cortex of the rat: a similar proportion of animals showed weak labelling of cells in the dorsal motor nucleus though many cells within the nodose ganglion were strongly labelled. These previous authors assumed that this was artefactual, and, in the case of Gattone *et al.* (1986), this is certainly possible since twice the volume of dye was injected. In the present work, however, we believe that a more likely explanation for the inconsistent labelling of motor nerve endings is their situation or number and we believe that a vagal efferent innervation of the adrenal gland in the rat does exist and that negative results reflect the presence of few and/or more peripherally situated endings (in the medulla or cortex).

In the guinea-pig, however, with some 120 efferent neurons with perikarya in the dorsal motor nuclei being strongly labelled, except after vagotomy, there is no question that the adrenal gland receives a significant vagal visceral efferent innervation.

In speculating about the possible sites of endings and targets for the visceral efferent innervation of the adrenal gland and the different findings in guinea-pig and rat, differences in functional activity and cellular composition of the glands may present clues. Three main differences immediately spring to mind: (i) the adrenal cortex of the guinea-pig is substantially larger than that of the rat and secretes a different principal adrenocortical steroid, (ii) the guinea-pig adrenal medulla is devoid of typical noradrenaline-storing cells, adrenaline-storing chromaffin cells being the predominant type, (iii) the guinea-pig adrenal medulla contains considerably more adrenomedullary neurons than the rat (Coupland, 1965*a*); hence the richer vagal efferent innervation in the guinea-pig may reflect a preganglionic innervation of these medullary neurons. Although the ganglion cells present in the adrenal medulla of most species are usually assumed to be sympathetic neurons and, by implication, arise from local neural crest (equivalent to Somites 18–24 of the chick; Le Douarin, 1982) there is no absolute evidence for this and in addition there is good evidence for the participation of vagal neural crest (Somites 1–5) in the development of structures mainly derived from intermediate cell mass mesoderm (Le Douarin, 1982). In order to determine the sites of efferent nerve endings, antegrade nerve fibre tracing studies are now clearly essential while experimental embryological investigations involving techniques similar to Le Douarin's chick-quail chimera studies may provide a useful lead to the ontogeny and functional roles of the adrenal medullary neurons.

The findings of a vagal afferent innervation of the adrenal glands of rat and guinea-pig in the present study, taken together with previous evidence of a relatively rich spinal afferent innervation of the glands in the same species (Mohamed, Parker & Coupland, 1988; Mohamed, 1988 and Parker, Afework & Coupland, unpublished), suggest that afferent adrenal nerve fibres play an important role in local reflex control and in more general homeostatic mechanisms. It is, therefore, important to recall that physiological evidence for the existence of adrenal baro- and chemoreceptors was presented by Niijima & Winter (1968*a*, *b*) in cats and rabbits. In the guinea-pig recent findings (Mohamed, 1988) indicate that as judged by the number of perikarya labelled and spinal segments involved, the adrenal glands enjoy a similar degree of spinal motor and sensory innervation. Furthermore, since the injected label (FB or HRP) was mainly confined to the adrenal medulla it is likely that the majority of sensory endings lie within this part of the gland. However, tracer studies involving the injection of label into the sensory ganglia are needed to localise the sites of endings and these should be complemented by the chemical characterisation of the neurons involved.

In addition to the typical afferent autonomic sensory role involving possibly chemoreceptors and mechanoreceptors the relatively rich sensory supply of the gland may be of importance in respect of neurogenic catecholamine secretion (Khalil, Livett & Marley, 1986), substance P possibly being released at sensory nerve endings within the adrenal medulla. In this context it is interesting to note that Lindberg *et al.* (1979) and Gilbert *et al.* (1980) demonstrated rapid axonal peripheral transport of substance P, vasoactive intestinal peptide and somatostatin in the cervical vagus nerve in the rat.

Unlike spinal afferent and efferent neurons distributed to the adrenal gland, which are strictly ipsilateral, vagal fibres originate bilaterally in both the vagal sensory ganglia and the dorsal motor nucleus of the vagus; a bilateral vagal innervation was also observed in the kidney (Gattone *et al.* 1986; Wyss & Donovan, 1984; Donovan *et al.* 1983).

The results obtained after unilateral vagotomy in the present series show a difference between rat and guinea-pig in the position of crossing of fibres. In the rat the fibres clearly cross the midline below the lower cervical region – probably in the oesophageal and/or pulmonary plexuses. In the guinea-pig the crossing fibres must travel in a branch or branches of the vagus which leave the main trunk above the level of the vagal section (junction of upper two thirds and lower one third of neck) and travel caudally towards midline viscera reaching the visceral nerve plexuses in either the lower third of the cervical region or within the thorax before crossing to join a branch of the contralateral vagus in or below the lower third of the neck. Cervical, cardiac, pulmonary or higher oesophageal branches of the vagus and the associated plexuses would, therefore, seem to be the most likely elements involved, and dissection of the guinea-pig cervical vagus demonstrates appropriately directly cervical branches (Coupland, unpublished).

SUMMARY

Following the injection of 3–5 μl of horseradish peroxidase or Fast Blue into the left adrenal medulla, labelling of neurons in the vagal sensory ganglia of rats and guinea-pigs has been demonstrated in all cases. Labelled vagal motor neurons with cell bodies in the dorsal motor nucleus of the vagi have been demonstrated in all cases in the guinea-pig and occasionally in the rat. The possible reasons for this variation are discussed.

In both rat and guinea-pig the motor and sensory vagal innervation of the rat adrenal is derived from bilaterally situated cell bodies and the proportion of innervation derived from the two sides is approximately equal but with a slight ipsilateral predominance.

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