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THE LOCATION OF CARDIAC VAGAL PREGANGLIONIC NEURONES IN THE BRAIN STEM OF THE DOGFISH *SCYLIORHINUS CANICULA*

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SUMMARY

The locations, within the brain stem, of vagal efferent preganglionic neurones with axons in the two pairs of cardiac vagal rami of the dogfish have been defined by the retrograde intra-axonal transport of horseradish peroxidase (HRP). HRP was applied to the cardiac rami in one of two ways: either as crystals placed on the cut central end of the nerve or as a dried concentrated solution administered into the nerve on the tip of a fine pin. No difference was observed in the number of labelled cell bodies identified using either method. Labelled branchial cardiac vagal motoneurones were found ipsilaterally in the medial division of the vagal motor column, in the lateral division of the vagal motor column, and scattered between these two locations. In contrast, visceral cardiac vagal motoneurones were confined to the ipsilateral medial division of the vagal motor column. We suggest that the dual location of cell bodies supplying axons to the branchial cardiac branch of the vagus may represent a separation of function with respect to the two types of activity conducted by this nerve. Cardiac efferent fibres are confined in their exit from the brain to a middle group of vagal rootlets. This corresponds to the topographical representation of cardiac efferent somata within the extent of the vagal motor column.

INTRODUCTION

Elasmobranch fish are unusual amongst vertebrates in that nervous control of the heart is mediated solely by inhibitory tone exerted *via* branches of the vagus nerve. There is no accepted physiological evidence for sympathetic innervation of the heart of elasmobranchs (Young, 1933; Burnstock, 1969; Short, Butler & Taylor, 1977).

In the dogfish *Scyliorhinus canicula* the vagus nerve leaves the brain as a series of rootlets which are compressed together as they pass through the chondrocranium. Most proximally the vagus divides to form branchial branches 1, 2, 3 and 4, which contain motor fibres innervating the intrinsic respiratory muscles of gill arches 2, 3, 4 and 5 respectively, the 1st gill arch being supplied by the IXth (glossopharyngeal) nerve. The heart is supplied by two distinct pairs of vagal rami composed entirely of myelinated fibres (Short *et al.* 1977). The branchial cardiac branches arise from the

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fourth branchial branches of the vagus (Norris & Hughes, 1920) and the visceral cardiac branches from the visceral branch of the vagus (Marshall & Hurst, 1905). The cardioinhibitory action of these cardiac vagi has been well described (Bottazzi, 1902; Lutz, 1930; Von Skramlik, 1938). The branchial cardiac branches are more effective in slowing the heart than the visceral cardiac branches (Short *et al.* 1977; Taylor, Short & Butler, 1977). No evidence for an inotropic influence on the heart has been reported for either of the two pairs of cardiac vagi.

Practically nothing is known about the position of the neuronal sub-populations of the vagal motor column that innervate specific target organs in elasmobranchs (Smeets, Niewenhuys & Roberts, 1983), although the gross location of the vagal motor column has been described, mainly in normal stained material, in a number of species (e.g. Black, 1917; Ariëns Kappers, 1920; Smeets & Niewenhuys, 1976; Smeets *et al.* 1983).

In the present study we have determined the distribution of preganglionic neurones giving rise to both pairs of vagal rami that innervate the heart of the dogfish. In addition the exit of cardiac efferent fibres from the brain was shown experimentally to be confined to the middle vagal rootlets. A preliminary account of part of this work has been published (Barrett, Roberts & Taylor, 1983).

METHODS

Retrograde labelling with horseradish peroxidase (HRP)

The results of this investigation were obtained from 13 dogfish, *Scyliorhinus canicula*, of either sex, with body length between 55 and 66 cm and supplied by the Marine Biological Association, Plymouth where the experiments were performed. The fish were maintained in large tanks of circulating sea water for at least 1 week before the start of the experiments. Prior to surgery each fish was anaesthetized with MS 222 dissolved in aerated sea water (Sandoz, $0{\cdot}17\,g\,l^{-1}$). When ventilatory movements became weak (after approximately 20 min) the fish was placed on an operating tray, covered with crushed ice, and left to cool for 5 min (Williamson & Roberts, 1981). The cardiac branches of the vagus were exposed on the floor of the anterior cardinal sinus (Short *et al.* 1977; Taylor *et al.* 1977), by making a 5-cm incision through the skin forward from the anterior margin of the pectoral girdle, 1 mm below and parallel to the lateral line. A cut perpendicular to the body axis was then made through the underlying muscle to the roof of the cardinal sinus which was opened to expose the required branch of the vagus nerve.

HRP (Sigma, type VI) was applied in one of two ways. In the first set of experiments (seven fish) the selected branch of the vagus was cut peripherally as it entered the ductus Cuveri, freed from connective tissue and lifted clear of the blood. The central cut end of the branch was placed on a small non-absorbent plastic strip and surface moisture removed by blotting. A few crystals of HRP were placed on the cut end of the branch and the preparation was left for 30 min. During this time the blood in the sinus was kept below the level of the severed nerve by manipulating the position of the anaesthetized fish. After 30 min, excess HRP was removed and a small amount of petroleum jelly applied around the cut end of the branch before replacing it on the floor of the sinus.

In the second set of experiments (six fish) the branch of the vagus was not cut peripherally. A concentrated solution of HRP was allowed to dry on the tip of a steel entomological pin (size A3). Approximately 2 mm of the required branch of the vagus was cleared of connective tissue. The cleared piece of nerve was carefully lifted above the level of the blood, dried and then punctured with the tip of the pin containing the dried HRP solution. The pin was then gently pushed 2 mm along the nerve towards the brain and was left in position for 30 s before being carefully withdrawn. To ensure that as many axons as possible were damaged, and allowed contact with the HRP, this procedure was repeated at least three times before a small amount of petroleum jelly was applied over the puncture in the nerve.

The post-operative survival (fish surviving until the processing date) was enhanced in those fish in which the pin technique was used. The pin technique involved the opening of the anterior cardinal sinus for less than 10 min (rather than for approximately 45 min which was necessary when using the other method) thus the danger of air being aspirated into the ductus Cuveri was greatly reduced. The pin technique also minimized the danger of HRP entering other nerves, damaged during surgery, which could lead to erroneous conclusions on the central origin of particular motoneurones (Kidd & McWilliam, 1979).

After the administration of HRP into the nerve the wound was sutured and covered with a patch of thin rubber membrane attached with cyanoacrylate adhesive. Each fish was then kept in a large recovery tank at 16–19 °C, for 4–10 days, depending on the temperature of the water, the body length of the fish and the length of the branch of the nerve from the point at which HRP had been applied to its junction with the brain.

When a fish was deemed ready for processing it was placed in sea water containing MS 222 (0·17 g l^{-1}) for 30 min. The fish was then injected with 1 ml of alphaxalone-alphadolone (Althesin, Glaxo Laboratories) *via* the sub-orbital sinus. The anaesthetized fish was perfused through the heart, first with dogfish Ringer solution for 3 min (during which time a further 1 ml of Althesin was injected), then with a mixture of 50 ml 25 % glutaraldehyde, 28·5 ml Analar formalin and 921·5 ml phosphate buffer (pH 7·4) as a fixative, for 20 min (Mesulam, 1978). The brain with surrounding cartilage was dissected out and placed in the fixative for approximately 4 h. The preparation was then dissected further until only the cerebellum, hindbrain and a small part of the spinal cord remained. This was placed in fresh fixative and left overnight at 4 °C. The following morning the preparation was transferred to cooled sucrose buffer and left for approximately 3 h prior to sectioning. Serial frozen sections were cut in the transverse plane at a thickness of 60 μm using a sledge microtome with a freezing platform.

Free-floating sections were reacted using tetramethylbenzidine (TMB) (Sigma) as the chromogen (Mesulam, 1978); 4 drops of 0·01 mol l^{-1} cobalt chloride were added at the start of the pre-reaction soak, and 3·5 ml of 0·3 % hydrogen peroxide (the 'incubation solution') added at the end of the pre-reaction soak. The observed cell bodies were accurately drawn, using a *camera lucida* attached to a Leitz microscope and the area of cells, in which the nucleus was clearly visible, measured from the drawings using a modified planimeter (Summagraphics). Photomicrographs were taken with a 35 mm Nikon camera attached to a Leitz microscope. The distribution of the labelled cell bodies was mapped with reference to the sulcus medianus inferior and obex.

The identification of vagal rootlets containing cardiac motor fibres

The investigation used four dogfish of either sex with body length between 58 and 62 cm. Each fish was anaesthetized in sea water containing MS 222 ($0{\cdot}17\,g\,l^{-1}$) and packed in ice during surgery. After decerebration the hindbrain was exposed as in the subsequent paper (Barrett & Taylor, 1985*b*). Two fine copper wires coated with varnish, with approximately 2 mm of the tips exposed, were inserted close to the pericardial cavity and held in position by a patch of thin rubber membrane secured to the skin of the fish with cyanoacrylate adhesive. These wires were attached to a pre-amplifier (Isleworth, type A101) and the ECG signal displayed on a pen recorder with rectilinear coordinates (Devices M19). The fish was then transferred to the experimental tank where it was clamped into a stereotaxic frame (Narishige Instruments) with a plate inserted into the mouth and clamped dorsally, and another lateral clamp holding the body of the fish posterior to the pectoral fins and dorsal to the midline to avoid constriction of the posterior cardinal sinus (Taylor & Butler, 1982). The gills were force ventilated with aerated sea water *via* a tube inserted into the mouth below the clamp. The fish was allowed 2 h to recover from the effects of anaesthesia before the start of the experiment. Tubocurarine (Tubarine, Wellcome) was then injected

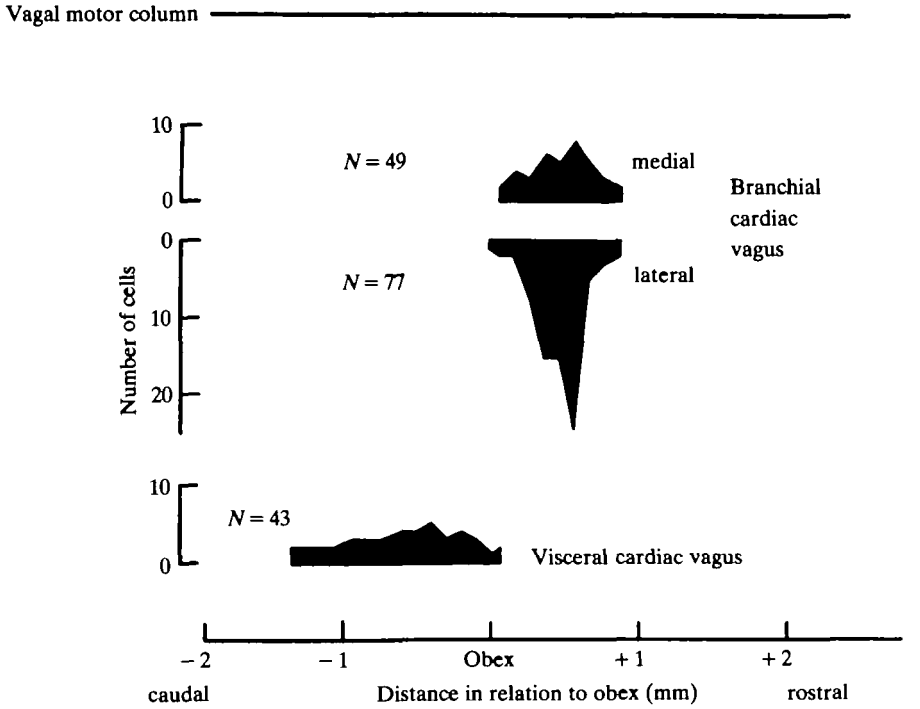

Fig. 1. A diagrammatic representation of the rostrocaudal distribution either side of obex of the visceral and branchial cardiac vagal motoneurones. The extent of the vagal motor column (top line) is taken from Barrett, Roberts & Taylor, 1984. Labelled cell bodies were counted (at 120-μm intervals) from the best backfills of each branch in single preparations. Labelled branchial cardiac motoneurones were found in two locations (medial and lateral); N is the number of cells identified in each location or, throughout the extent of the distribution (visceral cardiac vagus).

into the left sub-orbital sinus (7·5 mg kg^{-1}) and the fish was left for a further 1 h. The vagal rootlets on one side of the fish were severed and following 30 min recovery the vagal rootlets on the opposite side of the fish were cut sequentially, at 10-min intervals, just peripheral to the brain, either starting from the rostral or the caudal end of the brain. Heart rate was monitored continuously throughout the experiment.

Whenever possible measured variables are given as mean values ± S.E.M. with the number of observations in parentheses.

RESULTS

No difference was evident, in the number of labelled cells identified, between the two methods used for the application of the HRP into the branches of the vagus. For example, 42 visceral cardiac cell bodies were identified in one experiment using HRP applied to the central cut end of the nerve, and 43 visceral cardiac cell bodies were identified in an experiment using HRP applied on a pin into the intact nerve.

Cardiac vagal motoneurones

HRP was successfully applied to the branchial cardiac branch in nine fish and to the visceral cardiac branch in four fish.

Labelled visceral cardiac motoneurones were found in the caudal part of the vagal motor column from 1·06 mm caudal to 0·06 mm rostral to obex (Fig. 1) positioned medially close to the lateral edge of the 4th ventricle (Fig. 2). The rostrocaudal extent of their distribution (1·12 mm) belies the relatively small number of labelled cells. The maximum number of cells found in a single 60-μm section was seven, and

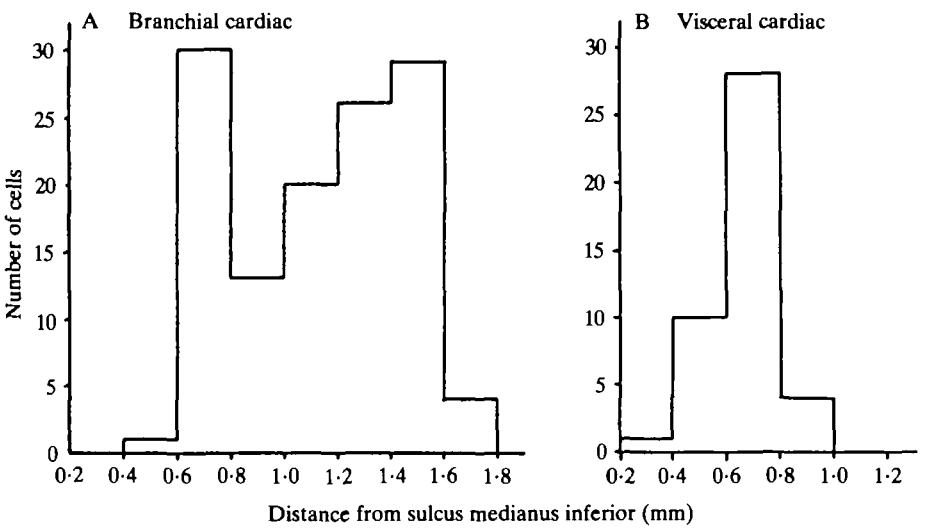

Fig. 2. Histograms of the mediolateral distribution in the hindbrain of labelled cell bodies from the best HRP backfills of the two cardiac vagi from separate preparations: (A) with axons in the branchial cardiac branch of the vagus and (B) with axons in the visceral cardiac branch of the vagus. Note the bimodal distribution of branchial cardiac motoneurones compared to visceral cardiac motoneurones.

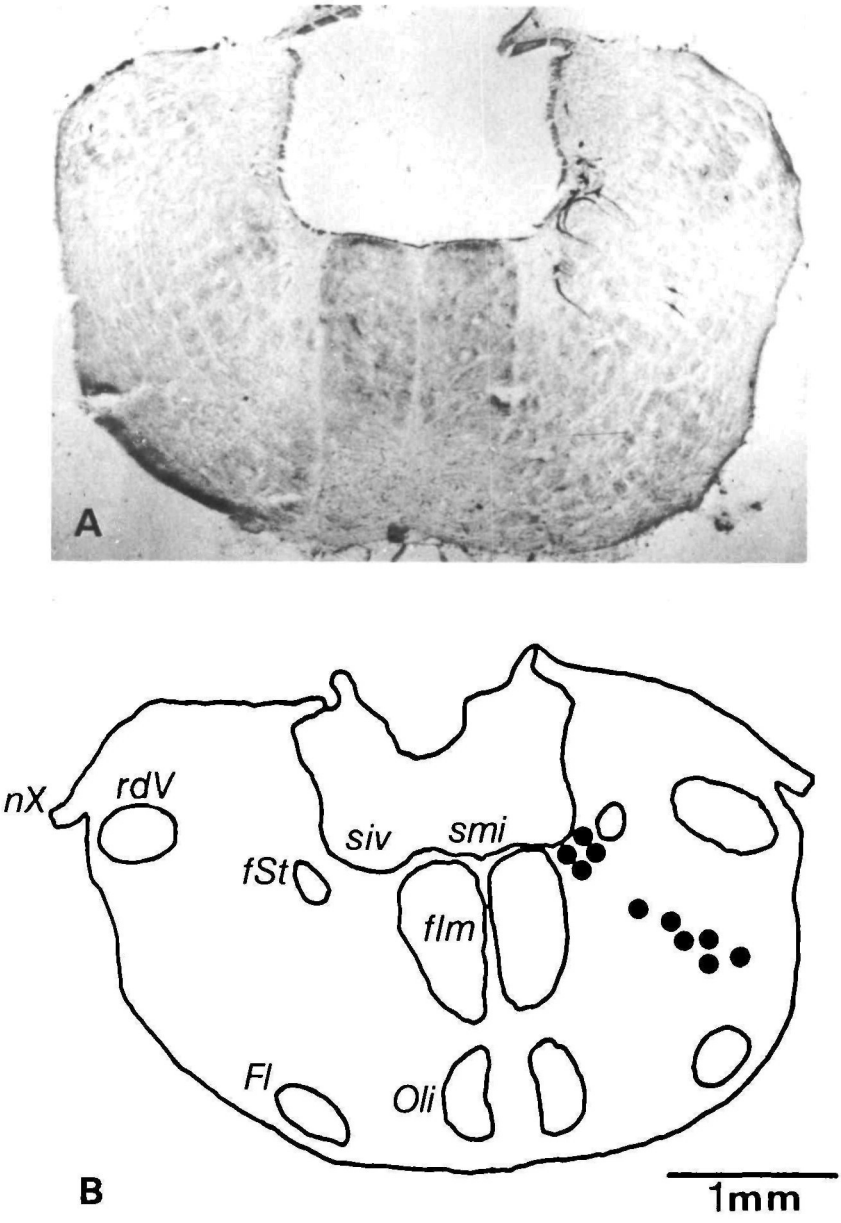

Fig. 3. (A) 60-μm transverse section through the hindbrain of a dogfish approximately 0·1 mm rostral to obex. Branchial cardiac motoneurones labelled with HRP are shown clearly on the right-hand side of the section. (B) A diagrammatic representation of a similar transverse section through the hindbrain to that shown in A. The locations of labelled branchial cardiac motoneurones(●) are shown in relation to other structures in the hindbrain *Fl*, nucleus funiculi lateralis; *flm*, fasiculus longitudinalis medialis; *fSt*, fasiculus medianus of Steida; *nX*, nervus vagus; *Oli*, oliva inferior; *rdV*, radix descendens nervi trigemini; *siv*, sulcus intermedius ventralis; *smi*, sulcus medianus inferior.

frequently only one or two labelled cells were identified (cf. Fig. 1). The number of labelled cells found from the application of HRP to the visceral cardiac vagus ranged from 17–43 cells in single fish.

Branchial cardiac motoneurones were found in the central part of the vagal motor column from 0·06 mm caudal to 0·78 mm rostral to obex (Fig. 1). The rostrocaudal extent of the branchial cardiac motoneurones (0·84 mm) was similar to the extent of the motoneurones supplying axons to each of the branchial branches (D. J. Barrett, B. L. Roberts & E. W. Taylor, in preparation) and was consistent with the fact that the branchial cardiac branch is the main post-trematic 4th branchial branch. Branchial cardiac neurones were found in the medial division of the vagal motor column close to the lateral edge of the 4th ventricle and also comprised a lateral division of the vagal motor column (Fig. 2). The spatial distribution of labelled cells characterized as either medial or lateral was continuous (Figs 2, 3) and both locations were occupied throughout the rostrocaudal extent of the branchial cardiac motoneurones (Fig. 1). The number of labelled cells identified ranged from 33 to 126 in a single fish. There was no obvious correlation between the number of labelled cells identified and the time the fish was left in the recovery tank or the length of the fish. The maximum number of cells found in a single 60-μm section was 18.

Branchial cardiac motoneurones overlap rostrally with motoneurones supplying axons to the 3rd branchial branch over a distance of approximately 330 μm. They overlap caudally with motoneurones supplying axons to the visceral branch of the vagus for most of their distribution (approximately 700 μm) (D. J. Barrett, B. L. Roberts & E. W. Taylor, in preparation).

The size of cardiac vagal preganglionic neurones

Cell area measurements were made of lateral and rostromedial branchial cardiac motoneurones from the same fish. The mean cell area of the lateral cardiac motoneurones was $741 \pm 39\ \mu m^2$ ($N = 45$), significantly different ($P < 0{\cdot}001 = t$-test) from that of the rostromedial cardiac motoneurones which was $650 \pm 36\ \mu m^2$ ($N = 34$). The mean cell area of the visceral cardiac motoneurones was not significantly different from that of the rostromedial branchial cardiac motoneurones.

Vagal rootlets containing cardiac motor fibres

In curarized preparations the control heart rate varied from 15 to 27 beats min^{-1} and a large increase was seen when all the vagal rootlets on one side were cut. The rate subsequently decreased to a value close to the control level. The rootlets on the other side of the fish were then cut sequentially in either a rostral or caudal direction.

The number of vagal rootlets that could be identified in each fish varied from 9 to 15. Wherever possible individual rootlets were cut, but the small size of some and their juxtaposition sometimes necessitated more than one rootlet being cut simultaneously. In fish in which 15, 11, 10 and 9 rootlets were identified, cutting rootlets 4–9, 4–8, 5–8 and 4–7 respectively, produced increases in heart rate. Cutting the other rootlets produced no significant change.

DISCUSSION

The branchial cardiac branch of the vagus receives axons from cells in two different locations in the medulla. Branchial cardiac motoneurones are found both rostromedially and solely comprise the lateral division of the vagal motor column (Barrett,

Roberts & Taylor, 1984). Cells are also found scattered between these locations. There is a morphological distinction between the two groups of cells, since the lateral cells are significantly larger than the two medial groups of cells.

In the elasmobranch *Squalus* an area lateral to the caudal part of the visceromotor column contains a distinct aggregation of large bipolar and triangular cells in Nissl, Klüver-Barrera and Bodian stained material (Smeets & Niewenhuys, 1976). These authors considered that this aggregation of cells represented a part of the motor nucleus of X and named it accordingly the nucleus motorius nervi vagi lateralis (Xml). The Xml extends from 2·00 mm rostral to approximately 4·0 mm caudal to obex. The vagal part of the visceromotor column was designated the nucleus motorius nervi vagi medialis (Xmm). The Xmm and the Xml, by virtue of their locations, may be the homologues of the mammalian dorsal vagal motor nucleus and the nucleus ambiguous respectively (Smeets & Niewenhuys, 1976). In *Squalus* the cells in the Xmm are somewhat larger than those in the Xml. These authors also described a medial vagal motor nucleus in *Scyliorhinus* but did not identify a lateral nucleus. The Xmm of *Scyliorhinus* extends from approximately 2·7 mm rostral to 2·25 mm caudal to obex (determined from the atlas provided by Smeets & Niewenhuys, 1976). More recently Barrett *et al.* (1984) have confirmed a similar rostrocaudal extent (4·83 mm) for the vagal motor column of *Scyliorhinus*.

It is interesting that of the two vagal rami that innervate the heart (the branchial cardiac and visceral cardiac branches) only motoneurones supplying axons to the branchial cardiac branch are found in two locations. The visceral cardiac motoneurones are confined to a medial position in the medulla. Peripheral stimulation of the cardiac branches in this species has indicated that the branchial cardiac branches were more effective in cardioinhibition than the visceral cardiac branches (Short *et al.* 1977) and selective transection of the branches indicated that the bradycardia during hypoxia was mediated predominantly *via* the branchial cardiac branches (Taylor *et al.* 1977). Recently, Taylor & Butler (1982) demonstrated that there are two types of nervous activity associated with the branchial cardiac branch; rhythmically bursting units, which are synchronous with ventilatory movements and sporadically active units. In the preceding paper it was shown that the bursting activity in paralysed fish is, at least partially, centrally generated (Barrett & Taylor, 1985*a*). It is suggested that the dual origin of branchial cardiac motoneurones may relate to the two types of nervous activity seen in peripheral recordings from this branch. Peripheral recordings from branchial branches of the vagus, which innervate the intrinsic respiratory muscles of the gill arches, contain predominantly rhythmic bursting units and respiratory vagal motoneurones are confined to the rostromedial division of the vagal motor column (Barrett *et al.* 1984). The medial branchial cardiac motoneurones could, by virtue of their location, be responsible for the rhythmically bursting units identified in peripheral recordings, whilst the lateral cells may be responsible for the sporadically active units that accelerate during hypoxia and possibly induce the reflex bradycardia (Taylor & Butler, 1982).

In the brain stem of some mammals the cell bodies of fibres which mediate the vagal bradycardia originate in the nucleus ambiguous (nA) (McAllen & Spyer, 1976, 1978). If the lateral division of the vagal motor column in *Scyliorhinus* can be regarded as the homologue of the nA then its functional role may be similar (Barrett & Taylor,

1985*a*,*b*). This possibility is supported by the observation that in the dogfish the branchial cardiac branches are more effective in cardioinhibition than the visceral cardiac branches (Short *et al.* 1977; Taylor *et al.* 1977) which arise solely from a medial location in the medulla.

It is interesting that, in the cat and the dog, the cell bodies of the cardiac motoneurones in the nA are larger than those in the dmnX (Geis, Kozelka & Wurster, 1981). This is similar to the situation in *Scyliorhinus* where the lateral cells (the nA homologue) are larger than the medial cells.

In *Squalus* which has 30 or more vagal rootlets, in contrast to the maximum of 15 identified in this study on *Scyliorhinus*, the majority are of mixed function, each comprising a wide sensory band and a narrow motor element (Norris & Hughes, 1920). This was confirmed for *Scyliorhinus* by cobalt backfills of the vagus nerve (Barrett, 1984), which indicated than an individual rootlet contained more sensory than motor fibres.

The rootlets containing cardiac motor fibres were found in the middle of the rostrocaudal distribution of the rootlets, a similar rostrocaudal location to that of the cardiac cell bodies in the vagal motor column (Barrett *et al.* 1984). Only cardiac fibres were shown to be localized in their exit from the brain in this study, but it seems plausible that the sequential representation of the peripheral distribution of the vagus in the vagal motor column may also be found in the exit of efferent fibres in the vagal rootlets, thus retaining some of the original morphological segmentation which is lost during development as the cranial segments fuse (cf. Young, 1950).

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