J. Anat. (1971), **110**, 2, pp. 203–213
With 10 *figures*
Printed in Great Britain

The use of cholinesterase techniques to study topographical localization in the hypoglossal nucleus of the rat

P. R. LEWIS*, B. A. FLUMERFELT† AND C. C. D. SHUTE*
Department of Anatomy, University of Cambridge

(*Accepted* 4 *August* 1971)

INTRODUCTION

The hypoglossal nucleus is perhaps the most suitable motor nucleus for the experimental study of the cytological changes occurring in cholinergic neurons following axotomy. The cells are large and the nucleus is easy to find even in a fresh unfixed brain; furthermore, the nucleus is so close to the midline that it is possible to use one side as a control for the other with complete confidence and to view equivalent control and experimental neurons simultaneously at quite high magnifications. An added advantage is that the hypoglossal nerve trunk in the neck region is almost purely motor; the central effects of axotomy are therefore not complicated by any significant loss of sensory fibres. Our interest in the nucleus was heightened by the discovery that in the rat a group of neurons at the caudal end contained a high concentration of an enzyme resembling in its histochemical reactions pseudocholinesterase (Shute & Lewis, 1963). The enzyme will hydrolyse acetylthiocholine and is inhibited by ethopropazine, but its most characteristic property is a rapid hydrolysis of butyrylthiocholine; BuChE would thus seem an appropriate abbreviation to distinguish it from true cholinesterase (AChE), the enzyme typically present in motor neurons.

It was shown originally by Schwarzacher (1958) that there is a marked decrease in AChE activity in hypoglossal neurons during the second and third weeks following axotomy (although he also looked at the response of pseudocholinesterase he did not comment on the specifically staining group of cells). It has since been shown that other cholinergic neurons show a similar response when their peripheral axons are sectioned (see Soderholm, 1965; Navaratnam, Lewis & Shute, 1968; Navaratnam & Lewis, 1970). The use of cholinesterase techniques therefore offers a clear-cut method of determining the central origin of motor nerves. This method is particularly valuable in the hypoglossal nucleus since the chromatolytic changes in these neurons are not well shown by conventional histological techniques.

* Present address: Physiological Laboratory, University of Cambridge.
† Present address: Department of Anatomy, University of Western Ontario, London, Canada.

MATERIALS AND METHODS

Albino rats of both sexes, and of ages ranging from 4 to 16 months, were used. In experimental animals one or more branches of the hypoglossal nerve were cut in the neck region. Between 10 and 20 days after nerve section, the animal was fixed by perfusion with 20 % formal saline through the aorta, and the brain was dissected out as quickly as possible. The hindbrain, with the cerebellum removed, was fixed for a further 4–6 h in 10 % neutral formalin at 4 °C and stored overnight in water, also at 4 °C. Approximately 1 h before sectioning, the tissue was transferred to 20 % aqueous alcohol to improve its cutting qualities. Sections were cut on a freezing microtome at thicknesses ranging from 25 to 100 μm and their serial order preserved in plastic haemagglutination trays.

The histochemical techniques employed to demonstrate cholinesterase activity were refinements of the thiocholine method introduced by Koelle & Friedenwald (1949). For light microscopy the procedure was essentially that described by Lewis, Scott & Navaratnam (1970). For the electron microscopic study of the hypoglossal nerve the procedure was that used by Lewis & Shute (1969), except that the concentration of glycine in the incubation medium was doubled and the incubation time increased, in order to give staining intense enough to be seen at low screen magnifications. An A.E.I. EM6B was used for the electron microscopy.

For acid phosphatase activity the method introduced by Gomori was used, as described in Pearse (1961): sections were incubated at pH 5·0 for 2 h at room temperature. For lactic dehydrogenase activity the procedure described by Pearse (1961, p. 911) was used: nitro-BT was used as the hydrogen acceptor and cyanide as the respiratory inhibitor; sections were incubated for 2 h at room temperature.

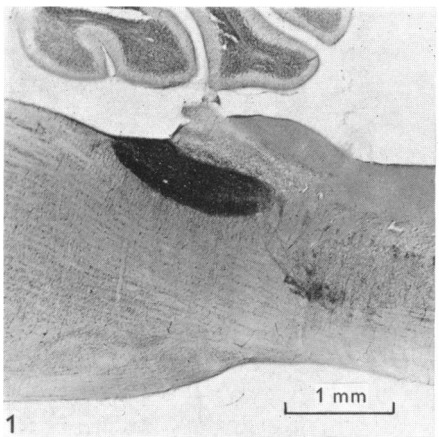
OBSERVATIONS

The hypoglossal nucleus in the rat forms a long column near to the midline of the medulla, beginning rostral to the decussation of the corticospinal tract, and extending as far as the cephalic pole of the dorsal vagal nucleus. Above the obex it occupies the central grey of the medial eminence which tapers caudally to form the hypoglossal trigone. At lower levels of the medulla it lies ventral to the central canal. Surrounding the hypoglossal nucleus is a ring of poorly defined nuclear groups which constitute the perihypoglossal nuclei. The remainder of the ventral aspect of the nucleus lies in relation to the medial longitudinal fasciculus.

The cells within the hypoglossal nucleus are similar in size and morphology to those in the ventral grey of the cord, being typically large, multipolar cells with coarse Nissl granules. The caudal part of the nucleus is divided into three distinct groups of cells on each side of the mid-line. Ventral to the central canal is found a compact cluster of cells which is the largest of the three groups. Dorsal to this cluster and separated from it by a narrow band is a smaller number of scattered cells which lie beside the central canal. The third group of cells is exceedingly small and lies ventral and lateral to the large cluster. This subgroup is separated from the other two by bundles of internal arcuate fibres which pass downwards to the decussation of the medial lemniscus. The ventrolateral cluster is only observed in sections through the

caudal part of the nucleus. Further forward, the nucleus becomes wider and the distinction between dorsal and ventral halves in normal material becomes less obvious.

When the rat medulla is stained by the thiocholine technique for AChE, the hypoglossal nucleus appears in sagittal section as a densely stained area which extends caudally from the floor of the fourth ventricle (Fig. 1). Reaction product is present in large amounts throughout the neurons and their processes, but this staining is obscured somewhat by large amounts of extracellular AChE. All the large neurons in the nucleus are intensely stained, including those of the ventrolateral cluster. Apart from the cells of the dorsal motor nucleus of the vagus, significant staining for AChE is not present in any area adjacent to the hypoglossal nucleus, which is therefore delineated far more clearly than by any ordinary histological technique. Clear spaces throughout the nucleus represent numerous blood vessels, which occasionally possess stained borders. Extending ventrally through the medulla are the hypoglossal nerve rootlets, which also stain for AChE.



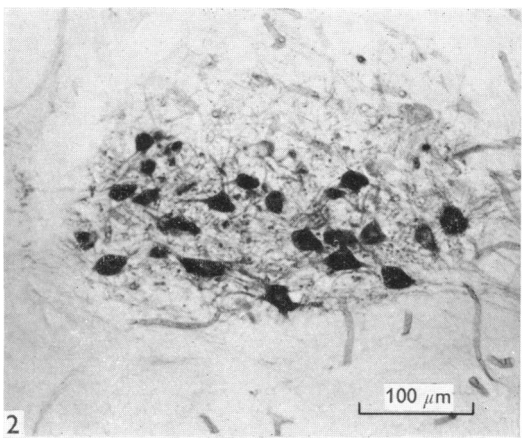


Fig. 1. A parasagittal section through the medulla stained for AChE to show the extent of the hypoglossal nucleus, which can be seen as an intensely stained band extending caudally from the floor of the fourth ventricle.

Fig. 2. A transverse section through the caudal end of the hypoglossal nucleus stained for BuChE to show the intense intracellular staining with relatively little extracellular staining.

When the tissue is stained for BuChE, the nucleus takes on a strikingly different appearance. Blood vessels form a heavily stained network of interwoven strands, but the neurons throughout the rostral part of the nucleus are completely devoid of reaction product. Some of the ventral cells in the caudal part of the nucleus, however, do possess marked BuChE activity and stand out distinctly because of the near absence of stain within the neuropil (Fig. 2). That these ventral cells possess both enzymes simultaneously was confirmed from serial sections in both the transverse and sagittal planes stained alternately for AChE and BuChE. In addition, frozen sections through the entire nucleus were carefully dissected along the midline and the two halves were stained separately for the two enzymes (Fig. 3). Since the marked

extracellular staining for AChE makes cells difficult to count, in some series a proportion of the sections were stained for acid phosphatase (Fig. 4).

The musculature of the tongue

The muscles of the tongue can be classified into those which cause the tongue to protrude and those which cause it to retract. It follows from the mode of attachment of the muscles that the protrusors consist of the genioglossus, geniohyoid and the transverse and vertical intrinsic muscles, while the longitudinal intrinsic muscles, hyoglossus, styloglossus and infrahyoid muscles make up the retractors.

The hypoglossal nerve, which supplies the tongue muscles, in the rat divides in the neck into a medial and lateral branch, the medial being the larger of the two. In serial sections of the floor of the mouth in young rats the medial branch of the hypoglossal nerve can be traced to the genioglossus and geniohyoid muscles and the lateral branch to the styloglossus and hyoglossus muscles. The distribution of the two branches to the different intrinsic muscles of the tongue cannot be determined.

In general, electrical stimulation of the medial branch causes the tongue to protrude and stimulation of the lateral branch causes it to retract. When the stimulation of the medial branch is subtetanic, the tongue is repeatedly protruded and then drawn back. Tetanic stimulation of this branch causes sustained protrusion with some deviation to the opposite side. Subtetanic stimulation of the lateral branch causes repeated retraction of the tongue and with tetanic stimulation there is extreme sustained retraction. These findings indicate that the fibres of the medial branch of the hypoglossal nerve are at least predominently distributed to protrusor muscles and those of the lateral branch to retractor muscles.

The central response to axotomy

To determine which portions of the nucleus give rise to the two main branches of the hypoglossal nerve, local changes in cholinesterase activity within the nucleus were observed following section of each branch separately. When the medial branch is transected a definite loss of enzyme staining is observed in the whole ventral portion of the nucleus, including the caudal cells containing BuChE (Fig. 5). When the lateral branch is cut the intensity of AChE staining diminishes in the rostral two-thirds of the dorsal portion: BuChE is not present in the dorsal part of the nucleus and shows no change when only the lateral branch is cut. The loss of enzyme in each case indicates the part of the nucleus which gives rise to the interrupted fibres. The time course of these enzyme changes will be reported in detail in a subsequent paper (B. A. Flumerfelt & P. R. Lewis, in preparation). It can be stated in general terms, however, that the loss of staining is obvious within 4 or 5 days of axotomy and remains so for at least a fortnight. The loss of BuChE is very striking (Fig. 5) and although the loss of AChE is never complete it is quite sufficiently marked throughout the second and third weeks to show which cells have been axotomized (Fig. 6).

Quantitative observations

The qualitative observations were extended by making cell counts in the nucleus and fibre counts in the nerve and its branches. The total number of cells was

estimated in two rats by counting neuronal nucleoli in serial transverse paraffin sections cut at 10 μm. The mean value obtained was 3484 ± 109 which can be rounded off to 3500.

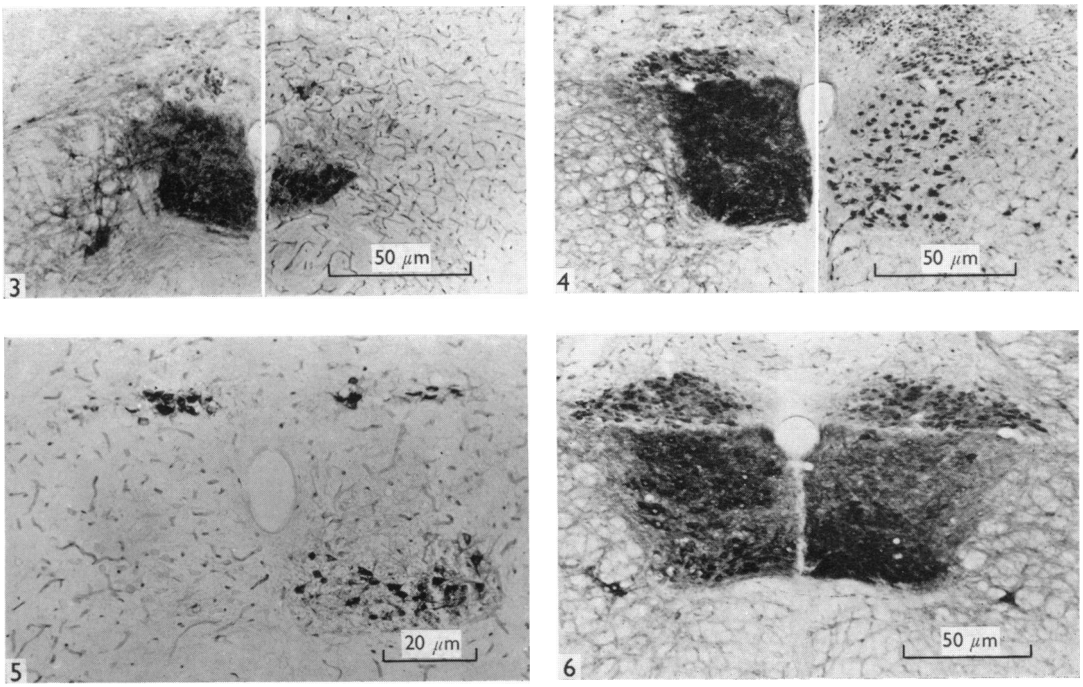


Fig. 3. Two halves of a transverse section through the caudal end of the hypoglossal nucleus, the left side stained for AChE and the right for BuChE. Note that the dorsal part of the nucleus and the small ventro-lateral cluster stain only for AChE, and that the main ventral part of the nucleus shows comparable staining for both enzymes.

Fig. 4. Two halves of a transverse section through the middle of the hypoglossal nucleus, the left side stained for AChE and the right for acid phosphatase. Note the large amount of extracellular staining for AChE and the clear delineation of the motor neurons by the acid phosphatase technique.

Fig. 5. A transverse section stained for BuChE through the caudal part of the nucleus from an animal in which the main hypoglossal trunk was cut on the left side 11 days beforehand. Note the almost complete loss of staining on the operated side. (The stained cells near the top of the photograph are in the dorsal motor nucleus of the vagus.)

Fig. 6. A transverse section stained for AChE through the middle part of the nucleus from an animal in which the lateral branch of the hypoglossal nerve was cut on the right side and the medial branch on the left side 11 days beforehand. There is obvious, though incomplete, loss of staining in the dorsal part on the right and from the ventral part on the left.

Cell counts were also made on transverse sections cut on the freezing microtome, and Fig. 7 shows the relative distribution of all neurons and of BuChE-containing neurons through the length of the nucleus. The results plotted in Fig. 7 were obtained from five normal hind brains which were sectioned and stained specifically for cell-counting. Each hind brain was cut at 50 μm and the sections placed alternately in two haemagglutination trays. The sections in one tray were stained for AChE and

were used to define the extent of the nucleus. The sections in the other tray were stained alternately for BuChE and for acid phosphatase. Thus, every fourth section through the nucleus was stained for BuChE and from this series the average number of stained cells per nucleus was estimated to be 427 ± 70. From the sections stained for acid phosphatase the average total number of all neurons was estimated to be 2462 ± 37. The latter figure was 30 % below the corresponding figure from the paraffin

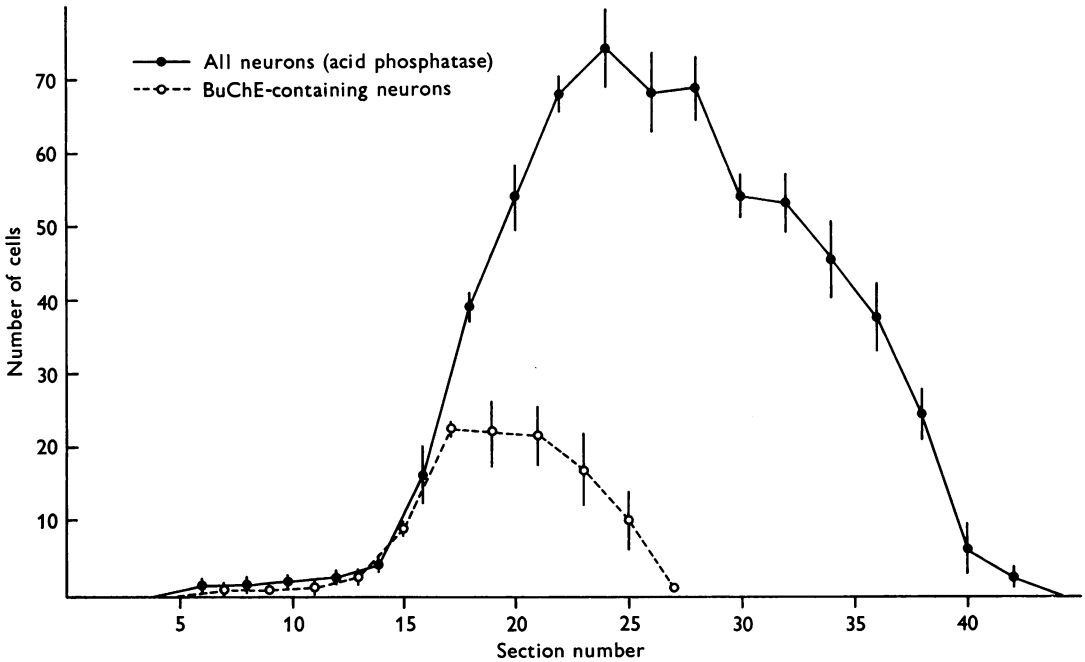


Fig. 7. Graph showing the relative distribution of neurons containing pseudocholinesterase through the length of the nucleus. Results obtained from five normal hind brains sectioned at 50 μm, in which one set of alternate sections from each brain was stained alternately for BuChE and for acid phosphatase. The plots of the cell counts from the individual brains were superimposed and counts for the five brains averaged. Each point represents the mean count $\pm$ the standard deviation. Most of the cells are concentrated between sections 15 and 40 (representing a length of 2·5 mm), with the BuChE-containing cells at the caudal end of the nucleus between sections 15 and 25.

series, a discrepancy which was probably due largely to the difficulty of making cell counts on the frozen sections. The appearance of the sections was very similar, however, after the two enzyme techniques, so a reasonably accurate value should be obtained for the overall percentage of neurons that contain BuChE. The value obtained from this series of five normal animals was $17{\cdot}3 \pm 2{\cdot}7$. A further series of five brains was sectioned at 30 μm and in two of these lactic dehydrogenase was used instead of acid phosphatase to obtain the total cell count. In these thinner sections the discrepancy compared with the paraffin series was much smaller (12·8 %) but the average percentage of BuChE-containing cells was not significantly altered at $17{\cdot}0 \pm 1{\cdot}2$. The most accurate estimate for the total number of cells in the nucleus is undoubtedly that obtained from the paraffin series; 17 % of that figure

is approximately 600 which therefore represents the best available estimate of the number of BuChE-containing cells in the hypoglossal nucleus on each side in our particular strain of rats.

In transverse Araldite sections the hypoglossal nerve is seen to consist largely of myelinated fibres measuring approximately 5 μm in diameter (Fig. 8). Some 3 % of the fibres are more heavily myelinated and measure about 12 μm in diameter. Unmyelinated fibres are very few in number. The total number of myelinated fibres was counted in the right nerve from three animals and the mean figure obtained was 3659 ± 32; the number of exceptionally large fibres was of the order of 100.

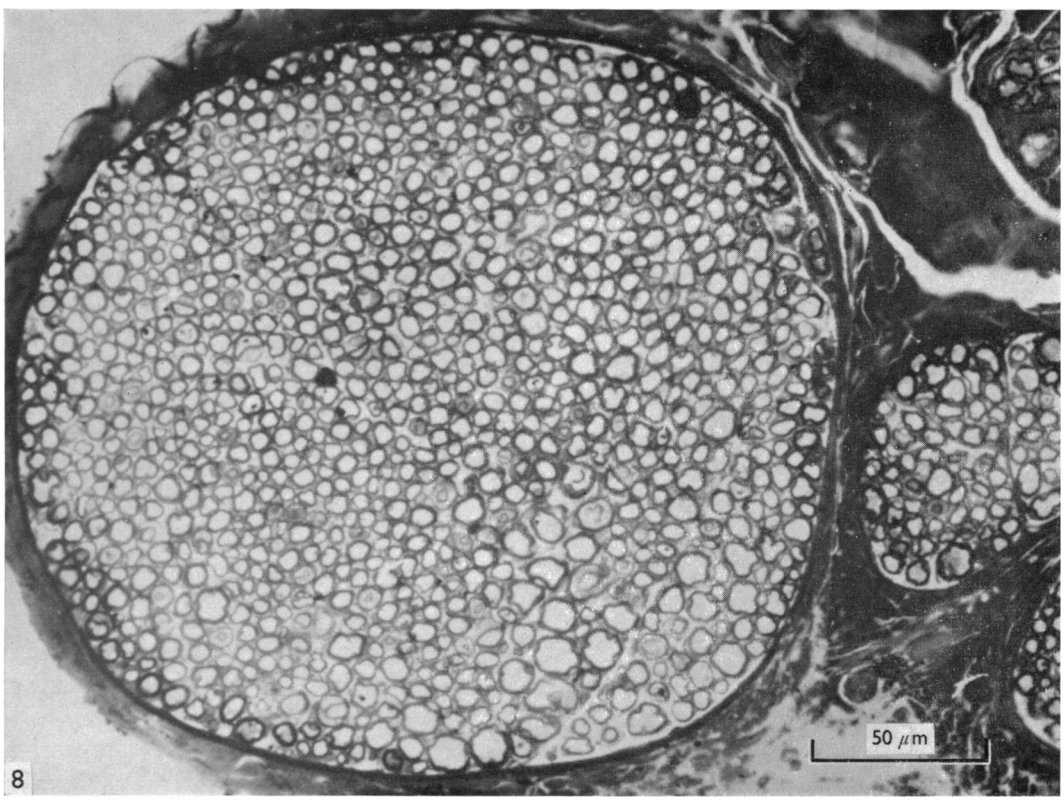


Fig. 8. A light micrograph of a 1 μm Araldite section through the lateral branch and some branchlets of the hypoglossal nerve. Mallory's azure II–methylene blue. Montage, × 460.

At the electron microscope level the presence of AChE activity in cholinergic fibres is revealed by an intense staining of the axolemma (Lewis & Shute, 1966, 1969). Within the hypoglossal trunk and its branches this characteristic appearance is observed throughout most of the fibres, although the largest fibres are usually devoid of reaction product (Fig. 9). The presence of BuChE along the axonal membrane is also observed in a proportion of fibres (Fig. 10) in the medial branch but not in the lateral one. Fibres which showed staining for BuChE in the main trunk were marked

off on a much enlarged (× 825) light micrograph from an adjacent 1 μm Araldite section. Rather more than 10 % contained a lighter, discontinuous deposit along the axolemma; recent experiments with a Philips 300 microscope have shown that such fibres often show unequivocal staining when the grid is tilted through a suitable angle. Hence, the number and proportion of hypoglossal fibres which stain for BuChE agree closely with the results of the cell counts on the nucleus.

DISCUSSION

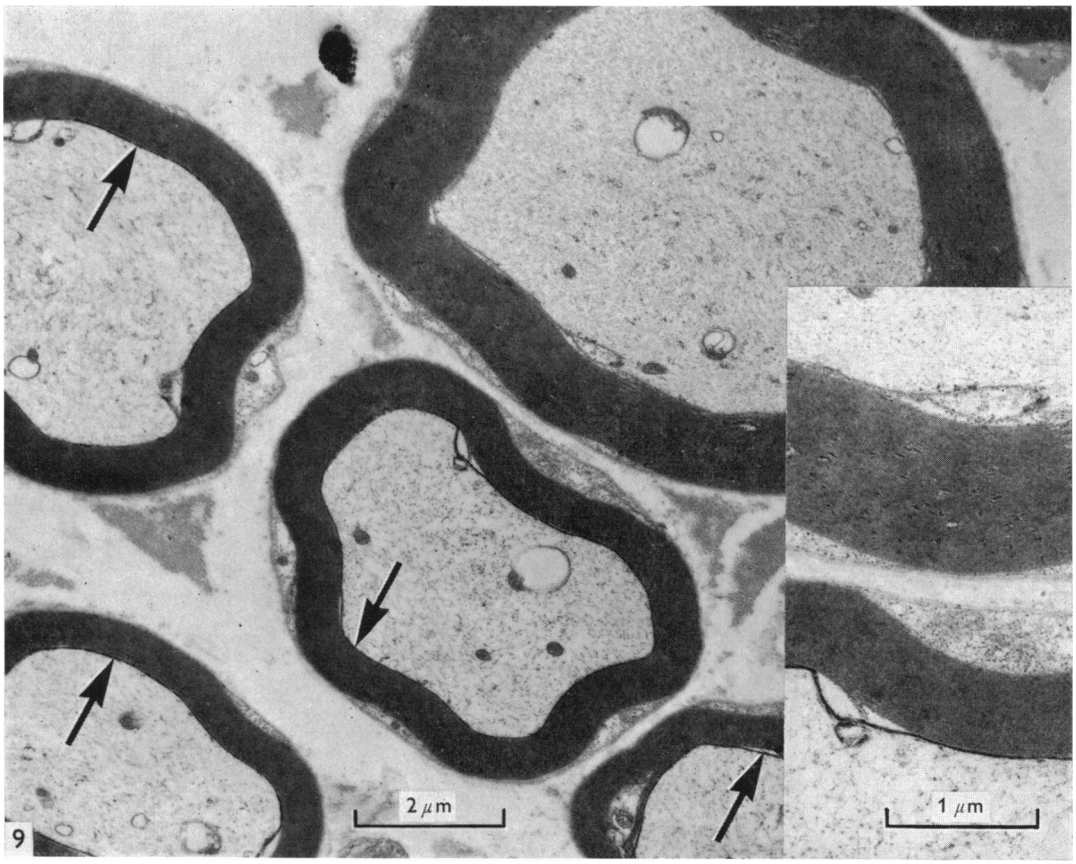
The thiocholine technique for cholinesterase clearly delineates the true extent of the hypoglossal nucleus. Thus the small caudal group of isolated cells lying ventrolaterally definitely consists of motor neurons supplying fibres to the medial branch of the hypoglossal nerve. Other groups of neurons lying outside the main area of staining almost certainly do not supply any motor fibres to the hypoglossal nerve.

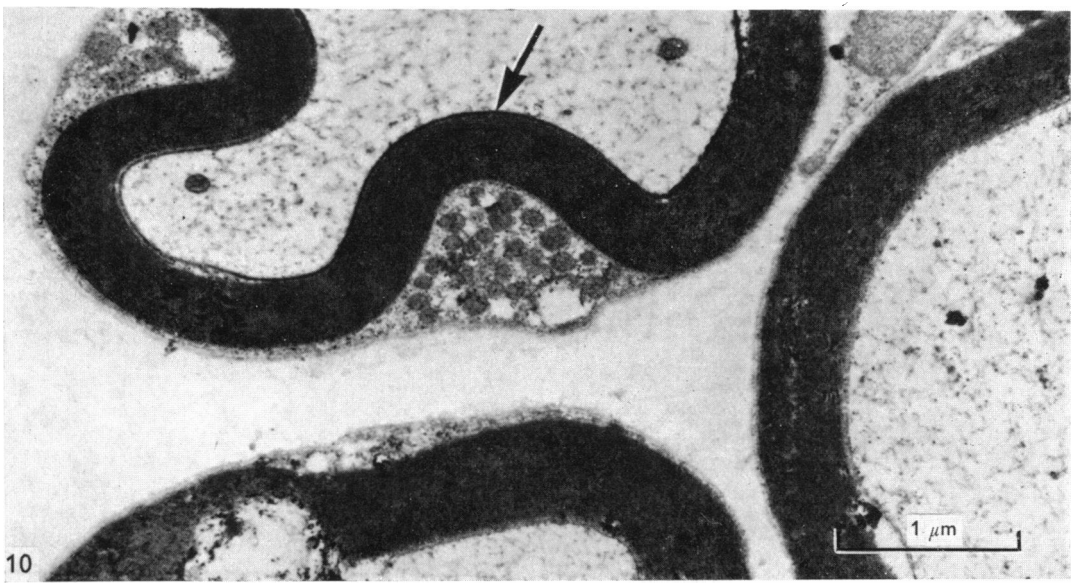
The sharp drop in cholinesterase staining shown by the neurons following axotomy makes the identification of the cells of origin of the branches of the hypoglossal nerve easy and unequivocal. This technique has also been successfully applied to the problem of the topographical distribution of neurons in the dorsal motor nucleus of the vagus supplying various branches of the vagus (Lewis *et al.*, 1970). A similar response to peripheral axotomy has been observed in all central cholinergic neurons that have been tested: in somatic motoneurons (Soderholm, 1965; Navaratnam & Lewis, 1970); in sacral autonomic neurons (Navaratnam & Lewis, 1970); and in neurons of the facial nucleus (C. C. D. Shute & P. R. Lewis, unpublished observations). The intensity and time course of the response may vary but it is always sufficient to be obvious in suitably stained sections. The technique should therefore have a wide application to problems of peripheral motor innervation. The fact that the response occurs following hypoglossal nerve section shows that deafferentation is not an important factor, since the hypoglossal nerve carries almost no afferent fibres – as was confirmed by the fibre counts on E.M. sections stained for cholinesterase.

As seen in cholinesterase preparations, the hypoglossal nucleus consists of dorsal and ventral parts approximately equal in size. It is not possible to recognize as distinct entities all the columns or subnuclei which have been described by Barnard (1940) and others. After section of the medial branch of the hypoglossal nerve, the whole of the ventral part loses its staining for AChE. Section of the lateral branch leads to loss of staining in the anterior portion of the dorsal part. These findings indicate that the ventral part innervates protrusor muscles and the dorsal part retractors. The posterior portion of the dorsal part presumably innervates retractors supplied by the main trunk of the hypoglossal nerve before its division into the two major branches, e.g. the infrahyoid muscles supplied by the descendens hypoglossi.

Fig. 9. From a transverse section of hypoglossal nerve stained for AChE. Note the intense staining of the axolemma (arrowed) in all the normal-sized fibres but not in the much larger fibre at the top right of the picture. The inset shows at higher magnification this difference in staining of the two types of fibre in the region of the internal mesaxon.

Fig. 10. From another transverse section of the same nerve stained for BuChE. Note that in only one of the three fibres (all of which are normal-sized) is the axolemma stained for BuChE (arrowed).


2 μm
1 μm
9


1 μm
10

Our results in the rat are thus in broad agreement with the conclusions of Stuurman (1916) and Barnard (1940) with regard to topographical localization within the hypoglossal nucleus of the mouse and dog respectively. These authors agree that the hyoglossus and styloglossus (retractor muscles) are supplied by cells in the dorsal part of the nucleus, and Barnard places the cells supplying the infrahoid muscles, via the descendens hypoglossi, caudally in the dorsal part.

The presence of high concentrations of BuChE in the ventro-caudal group of cells calls for special comment. Similar staining is seen in the rat central nervous system, notably in cells giving origin to preganglionic parasympathetic fibres – e.g. the dorsal motor nucleus of the vagus (Lewis *et al.*, 1970): many branchiomotor and somatic motor neurons contain only AChE. In the ventro-caudal neurons of the hypoglossal nucleus both enzymes have the same ultrastructural distribution in the endoplasmic reticulum and nuclear envelope (Lewis & Shute, 1965; Flumerfelt & Lewis, 1969); so it is likely that both enzymes have a similar function. It is significant that in the main trunk a proportion of the axons contain both enzymes and that this proportion is the same, within experimental error, as that of hypoglossal cells which stain similarly. The conclusion seems inescapable that these ventro-caudal neurons also contain both enzymes in their axons. Why these neurons should contain a second cholinesterase with an unusual specificity for choline esters is not obvious. The destination of their axons has not been proved experimentally, but it seems likely that they supply the intrinsic muscles of the tongue which cause its protrusion.

SUMMARY

The marked fall in cholinesterase content of a motor neuron that occurs within a week or so of axotomy has been used to study histochemically the topographical distribution within the rat hypoglossal nucleus. Thus it has been shown that the muscles which retract the tongue are supplied, via the lateral branch of the hypoglossal nerve, by motor cells in the dorsal part of the nucleus. The protrusor muscles are supplied by the ventral part of the nucleus. At the caudal end there is a ventral group of cells which possess both true cholinesterase and an enzyme resembling pseudocholinesterase: axons containing this same enzyme are present in the medial branch of the hypoglossal nerve. Counts of cells and axons have been made on histochemically-stained material to provide a quantitative picture of neuronal distribution in the nucleus.

We are most grateful to Mrs Anita Shelley for valuable technical assistance and to the Science Research Council for an equipment grant.

REFERENCES

BARNARD, J. W. (1940). The hypoglossal complex of vertebrates. *Journal of Comparative Neurology* **72**, 489–524.

FLUMERFELT, B. A. & LEWIS, P. R. (1969). Changes in the hypoglossal nucleus following axotomy. *Journal of Anatomy* **104**, 587.

KOELLE, G. B. & FRIEDENWALD, J. S. (1949). A histochemical method for localizing cholinesterase activity. *Proceedings of the Society for Experimental Biology and Medicine* **70**, 617–622.

LEWIS, P. R. & SHUTE, C. C. D. (1965). Changes in cholinesterase activity following axotomy. *Journal of Physiology* **178**, 25*P*.

LEWIS, P. R. & SHUTE, C. C. D. (1966). The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. *Journal of Cell Science* **1**, 381–390.

LEWIS, P. R. & SHUTE, C. C. D. (1969). An electron-microscopic study of cholinesterase distribution in the rat adrenal medulla. *Journal of Microscopy* **89**, 181–193.

LEWIS, P. R., SCOTT, R. A. & NAVARATNAM, V. (1970). Localization in the dorsal motor nucleus of the vagus in the rat. *Journal of Anatomy* **107**, 197–208.

NAVARATNAM, V. & LEWIS, P. R. (1970). Cholinesterase containing neurons in the spinal cord of the rat. *Brain Research* **18**, 411–425.

NAVARATNAM, V., LEWIS, P. R. & SHUTE, C. C. D. (1968). Effects of vagotomy on the cholinesterase content of the preganglionic innervation of the rat heart. *Journal of Anatomy* **103**, 225–232.

PEARSE, A. G. E. (1961). *Histochemistry: Theoretical and Applied* (2nd ed.) London: Churchill.

SCHWARZACHER, H. G. (1958). Der Cholinesterasegehalt motorischer Nervenzellen wahrend der axonalen Reaktion. *Acta anatomica* **32**, 51–65.

SHUTE, C. C. D. & LEWIS, P. R. (1963). Cholinesterase containing systems of the brain of the rat. *Nature, London* **199**, 1160–1164.

SODERHOLM, U. (1965). Histochemical localization of esterase, phosphatases and tetrazolium reductases in the motor neurons of the spinal cord of the rat and the effect of nerve division. *Acta physiologica scandinavica* **65**. Supplementum 256.

STUURMAN, F. J. (1916). Der Hypoglossuskern der Ameisenfresser. *Anatomische Anzeiger* **48**, 342–348.