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Title:

Use of Horseradish Peroxidase to Trace Nerve Cell Connections in Turtles

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An exogenous protein, horseradish peroxidase (HRP) was used to trace efferent neurons of the heart and retina in the turtle (Pseudemys scripta). Small doses of HRP were injected into the subepicardial tissue of the heart and after a 48-hour survival period HRP reaction-product accumulated in what appear to be neuron cell bodies along the vagus nerve. Control HRP injections into the external jugular vein did not label these cells, so a vascular route was ruled out.

After injection of HRP into the anterior chamber of the turtle eye, HRP reaction-product was observed in a group of cells which constitute a restricted nucleus in the midbrain. Most of the cells traced by this technique were in the nucleus on the side of the midbrain contralateral to the injected eye, but a smaller number of cells were traced to the ipsilateral nucleus.

USE OF HORSERADISH PEROXIDASE TO TRACE NERVE CELL CONNECTIONS IN TURTLES

A THESIS

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURES PAGE

INTRODUCTION

In this research, two separate questions were asked about the turtle nervous system:

1) What anatomical differences are there in the pathways of the two vagus nerves to the turtle's heart which might explain known differences in the effectiveness of these two nerves?

2) Does the turtle retina receive efferent fibers from the midbrain (which presumably would allow the brain to affect its sensory input), as is the case in some other vertebrates?

Both of these are basically anatomical questions which can be answered by neuron-tracing techniques. The technique chosen for these studies was a horseradish peroxidase (HRP) technique.

Background on question about vagus nerves

It has long been known (Bayliss, 1927; Garrey, 1912) that stimulation of the right vagus causes a complete stoppage of the heart, but stimulation of the left vagus causes only a slowing of the heart rate. By using neuronal tracing techniques, it might be possible to find anatomical differences between the right and left vagus nerves in the turtle which would explain the difference in response.

Background on question about retinal efferents

Efferent fibers to the avian retina were described first by

Ramon y Cajal (1889) and Perlia (1889). Using the method of retrograde degeneration Perlia found the source of these projections which was later described (Huber and Crosby, 1926) as the nucleus isthmo-opticus, located in the caudal part of the midbrain at the level of the trochlear nucleus.

Two recent studies which made use of HRP tracing techniques showed that both the chick and pigeon retina receive efferent fibers (LaVail and LaVail, 1972; Reiner and Karten, 1982). In both species the source of the fibers was the nucleus isthmo-opticus.

Efferent fibers to the retina have also been described for other vertebrate classes. Using the method of retrograde transport of HRP, Ferguson et al. (1978) have reported the existence of efferent fibers in a reptile (Caiman crocodilus). In this species these fibers are derived from a large oblong field of cells at the isthmic level of the midbrain, bounded medially by the trochlear nucleus and laterally by a group of cells they termed the nucleus isthmi.

Efferent fibers to the retina of a garter snake of the genus Thamnophis have been identified (Halpern et al., 1976). The source of these efferent fibers is the nucleus of the ventral supraoptic decussation, located medial to the ventral portion of the lateral rostral midbrain.

Background on technique which was utilized

During the second half of the nineteenth century, at a time when the foundations of neuroanatomy were being established, tracing neural connections required laborious dissections by hand. However, the development of silver stains (Ramon y Cajal, 1904; Bielschowsky, 1904) attracted many investigators into neuroanatomy. By using this technique, investigators were able to achieve progress in the clarification of neural connections. This technique dominated research until the beginning of the 1970's when neuroanatomists developed a newer, more versatile, powerful, and efficient technique to trace neural connections. This technique is based on axonal transport of an exogenous macromolecule, such as horseradish peroxidase (HRP), to the neural perikaryon.

There are four approaches by which HRP can be used to trace neural pathways. The first one is based on the attachment of a radioactive label to the HRP molecule so that by using autoradiography methods the distribution of the tissue-bound enzyme can be traced (Geisert, 1976). The second approach is based on covalent binding of the HRP molecule to a fluorescent substance which can act as a specific marker when the tissue is examined by fluorescence microscopy (Hanker et al., 1971; Norden et al., 1976). The third approach is based on the immunohistochemical detection of the enzyme through the formation of HRP-anti-HRP complexes (Vacca et al. 1975; Sofroniew and

Schrell, 1980). The fourth approach, which was used in this research, is based on the detection of a visible reaction-product which is produced by the enzymatic activity of the HRP (Straus, 1959).

The proper utilization of HRP in tracing neural connections begins with its uptake and transport within the nervous system. Once injected around a nerve ending, neurons can take it up from the extracellular fluid into axon endings and rapidly transport it back to their cell bodies. Subsequently, in a suitable medium, tissue-bound HRP will combine with its substrate hydrogen peroxide (H_2O_2) and the result is a [HRP·H_2O_2] complex. This complex will oxidize a chromogen added to the incubation medium, and the result is a dense colored precipitate used to mark the location of HRP. The reaction which takes place at sites containing HRP activity is summarized as follows:

$$HRP + H_2O_2 \longrightarrow [HRP \cdot H_2O_2]$$

$$[HRP \cdot H_2O_2] + AH \text{ , soluble chromogen, } \longrightarrow$$

$$(A) \downarrow \text{colored precipitate} + HRP + H_2O$$

MATERIALS AND METHODS

I. Animals

The animals used in this research were 350-400 gram red-eared turtles (Pseudemys scripta) obtained from Kons Biological Supply, Germantown, Wisconsin. 25 animals were used, 10 in the vagus nerve studies and 15 in the retinal efferent studies.

II. Procedures for vagus nerve studies

A. Injection

Turtles were anesthetized by intra-peritoneal injection with 3 cc of 10 % urethane. Once anesthetized, a 7 cm hole was drilled slightly anterior to the center of the plastron. Nine µl of 5 % solution of horseradish peroxidase (HRP) (Type VI, Sigma P-8375) was injected under the epicardium of the right ventricle of three turtles by means of a 100 ul syringe. In three other turtles, the same amount of HRP was injected under the epicardium of the left ventricle. The turtles were allowed to survive for 48 hours and then they were sacrificed.

B. Dissection

The plastrons were removed and the hearts were washed out with Ringer solution. In each case portion of the vagus nerve from the brain to the heart on the same side as the heart injection was removed for the next steps.

C. Fixation

The nerves were fixed separately for 30 minutes at 21-23

C in a mixture of 1.25 % glutaraldehyde and 1 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.2-7.4 (Rosene and Mesulam, 1978). Then they were transferred separately to a 30 % sucrose solution buffered at pH 7.2-7.4, and stored for 24 hours in a refrigerator at 4 C (Rosene and Mesulam, 1978). Both vagus nerves were then reacted with 3',3'-diamino-benzidine tetrahydrochloride (DAB) (Sigma D-5637) as described below in order to produce a brown reaction product (LaVail and LaVail, 1974).

D. Histochemistry

The right and the left vagus nerves were incubated separately for 20 minutes at 21-23 C in a medium containing 50 mg of DAB, 100 ml of distilled water, and 5 ml of 0.1 M phosphate buffer at pH 5.5. This was followed by an enzymatic reaction for another 20 minutes, which was initiated by adding 2 ml of 0.01 % hydrogen peroxide to the incubation solution (LaVail and LaVail, 1974). Each vagus nerve was passed through six rinses of five minutes each at 4 C in a postreaction solution of 0.005M phosphate buffer at pH 5.5.

E. Sectioning

Frozen sections were cut in a cryostat at thicknesses of 3-30 micrometers and picked up on slides. Some, but not all, of these sections were stained with neutral red. Both stained and unstained sections were rapidly passed through the following solutions:

Distilled water for 10 seconds

70 % ethanol for 10 seconds

95 % ethanol for 10 seconds

Two changes of 100 % ethanol for 10 seconds each

Two baths of xylene, 2-5 minutes each.

The slides were coverslipped with Permount and were examined under a light microscope.

F. Controls

Two turtles which had not been injected with HRP were sacrificed and run through the same histochemical and sectioning procedures as experimental animals to check for endogenous peroxidase and for certain other substances, such as hemoglobin and myoglobin, that by themselves are able to oxidize DAB in the presence of hydrogen peroxide (Nauta et al., 1974). An additional check was performed in two turtles to control for spread of HRP through the blood-vascular system. Each of these turtles received 9 ul of HRP via the external jugular vein. These animals were treated the same as the experimental turtles in terms of survival time, fixation, and histochemical and sectioning procedures.

III. Procedures for tracing retinal efferent fibers to their nucleus of origin

Procedures were the same as for the vagus nerve studies, with the following exceptions: 8 µl of 5 % HRP was injected into the

anterior chamber of the right eye of an anesthetized turtle. At 48 hours after the injection, the animal was reanesthetized, the skull was removed with forceps, and the whole brain was removed, washed in Ringer solution, and placed in fixative solution for 30 minutes. The whole brain was incubated in DAB, then sections were cut at 30 to 40 μm.

To control for spread of HRP to the midbrain via the blood vascular system, two additional turtles received 9 ul of HRP in the external jugular vein. Subsequent processing was the same as for the experimental animals.

RESULTS

I. Vagus

Retrograde transport of horseradish peroxidase (HRP) from the site of injection into the vagus nerves was studied in 6 turtles. Injection of HRP into the myocardium of the turtle right ventricle produced a result different from injection into the left ventricle.

Sections of the right vagus nerve showed the HRP reaction product, brown granules, accumulating within the cytoplasm of what appear to be neuron cell bodies (Fig. 1). The average number of these cells per section was 33 (sample of 6 sections from each of 3 animals). These cells were of two types: cells of the first type, which were predominant, were elongate (Fig. 2). They had nuclei, and axonal processes could be visualized under the microscope in most cells. These cells were 4 μm in their short axis and ranged in size from 9 to 17 μm in their long axis. Cells of the second type were spherical in shape (Fig. 3) and had nuclei, but their axonal processes were not visible in all cells. These cells ranged in size from 9 to 17 μm.

Sections of the left vagus nerve showed the same two types of cells as those of the right vagus nerve. However, the average number of these cells per section was 16 (6 sections in each of 3 animals at the same level as for the right vagus nerve).

HRP reaction product in these cells took the form of numerous small brown granules distributed fairly evenly within the

cytoplasm (Fig. 4). In some cells, HRP reaction-product extended into the axonal processes (Fig. 5).

The intensity of staining varied considerably from cell to cell, with respect to the number of granules present and the optical density of individual granules (Fig. 6). This was true even of cells in the same tissue section.

One set of controls (two turtles) had the object of checking to be sure the results were not due to endogenous peroxidase activity in vagus cells. In this control, the animals were not injected with HRP but subjected to the same fixation and sectioning procedures as the experimental animals. No reaction product was detected in either the right or the left vagus nerve sections except in erythrocytes, which are easy to identify.

Another set of controls (two turtles) had the object of ruling out entry of HRP into vagus cells via a vascular route. In this control, animals were injected with HRP into the external jugular vein and subjected to the same fixation and sectioning procedures as the experimental animals. There was no accumulation of HRP reaction-product in either the right or the left vagus nerve (Fig. 7). On the other hand, erythrocytes accumulated HRP reaction-product, and so did muscle cells (which were taken from the same animal and wrapped around the nerve for the practical purpose of providing a larger tissue mass for easier sectioning).

Figure 1. Photomicrograph of 30 μm cross section of vagus nerve showing what appear to be neuron cell bodies within the nerve. Arrows point to several individual cells. Magnification 583 X.

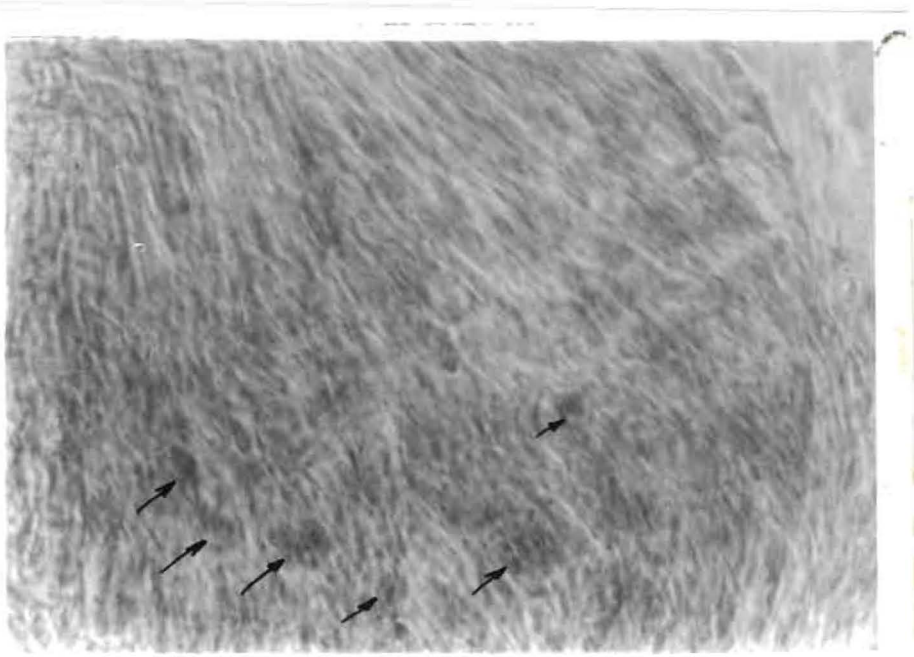


Figure 2. Photomicrograph of 30 μm cross section of right vagus nerve showing an elongate cell found within the vagus nerve. Granules in the cytoplasm are HRP reaction-products. Magnification 1458 X.

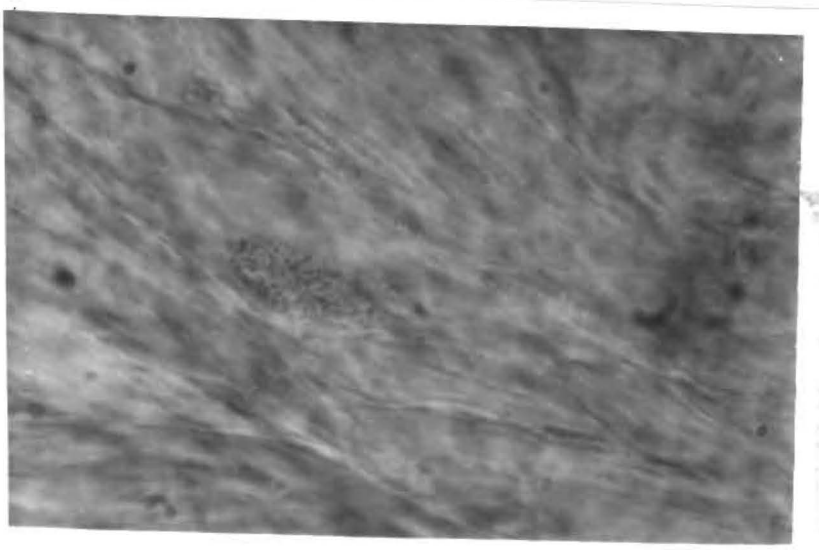


Figure 3. Photomicrograph of 30 µm cross section of the left vagus nerve, showing a spherical cell found within the vagus nerve. Granules of HRP reaction-product are visible in the cytoplasm. Magnification 1750 X.

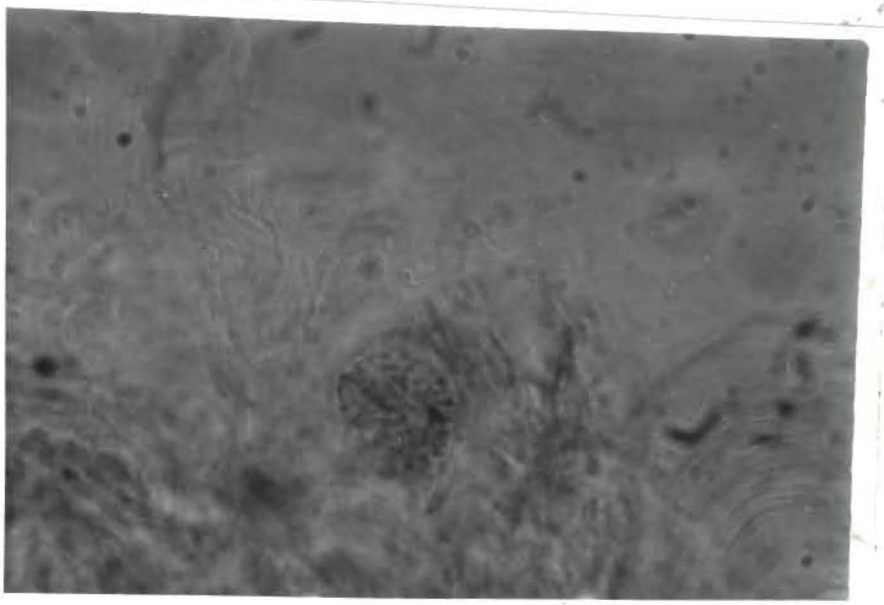


Figure 4. Photomicrograph of 8 μm cross section of the right vagus nerve, showing small granules of HRP reaction-product distributed within the cytoplasm of what appears to be a neuron cell body. Granules could be seen by eye in the dendritic branches but could not be brought into critical focus for photography. Magnification 1458 X.

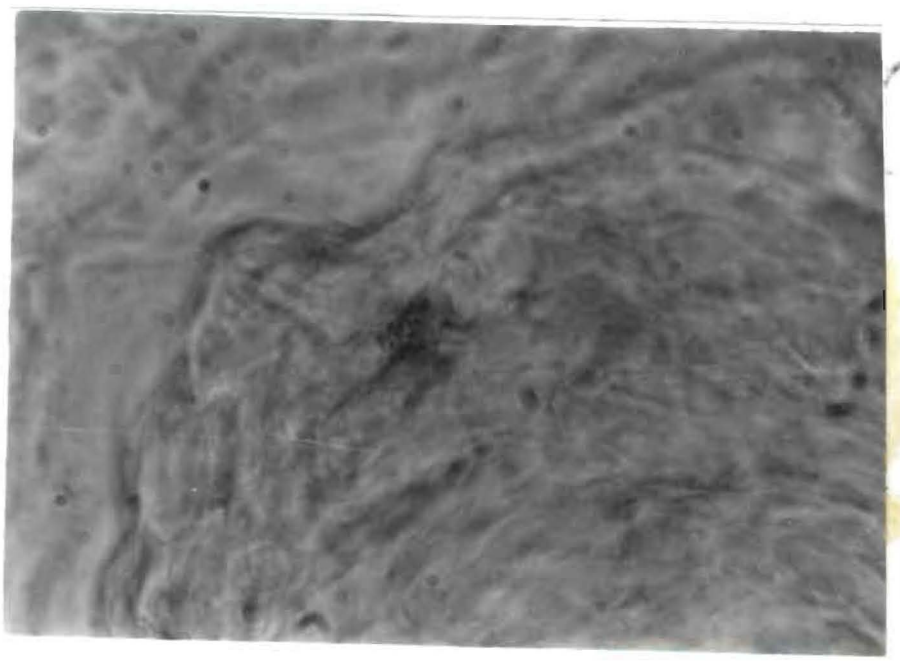


Figure 5. Photomicrograph of 30 μm cross section of the vagus nerve showing HRP reaction-product in the cytoplasm of a cell. Arrows indicate an axonal process containing granules which could be seen by eye but not photographed. Magnification 1458 X.

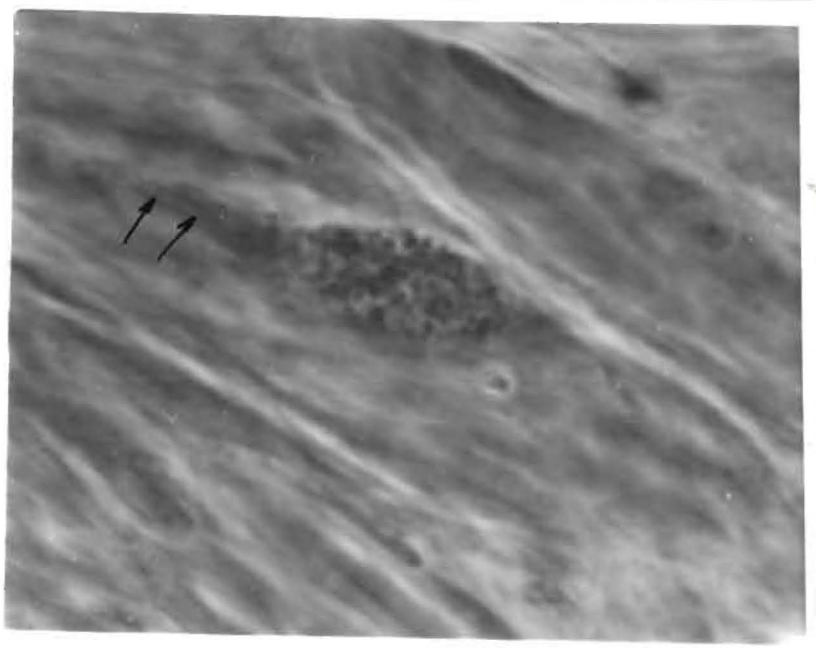


Figure 6. Photomicrograph of 30 um cross section of the vagus nerve showing strong and weak accumulation of HRP reaction-product. Cell at lower right contains obvious, dark granules. In the two cells at the top of the micrograph, lightly-stained granules could be seen by eye but did not show up well in the photograph. Magnification 583 X.

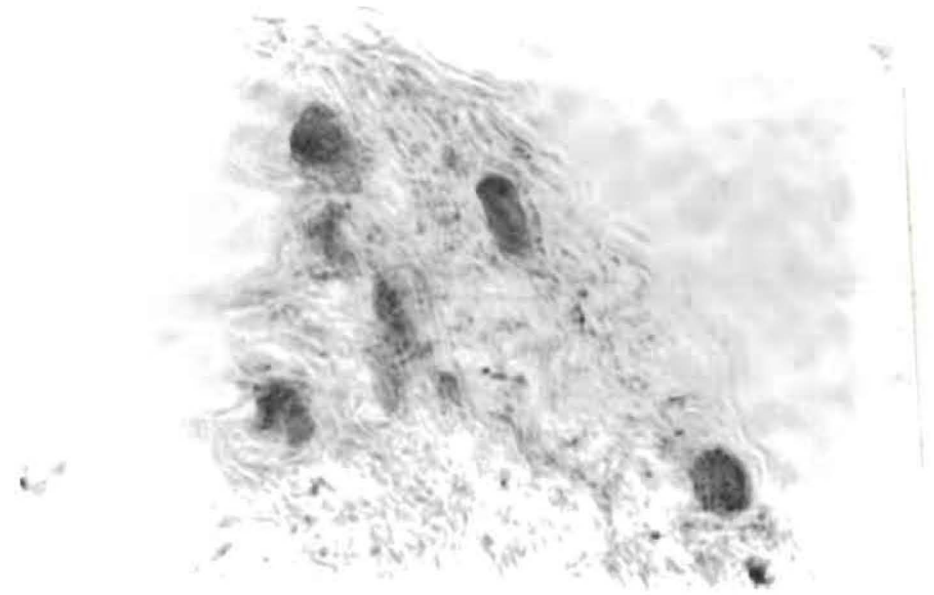
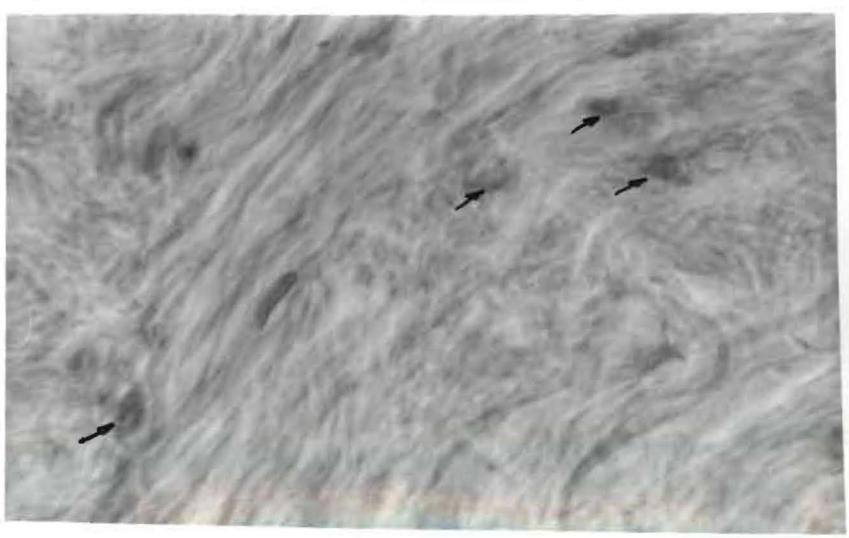


Figure 7. Photomicrograph of 30 μm cross section of the vagus nerve for control animal whose heart was not injected with HRP. This photomicrograph shows no HRP reaction-product within the cytoplasm of the cells and none was visible by eye. Several cells are marked by arrows. Magnification 583 X.



II. Retina

Injection of HRP into the anterior chamber of the eye in 13 turtles resulted in retrograde transport of HRP from the retina into the cell bodies of a restricted nucleus ventral to the optic lobes in the contralateral midbrain (see diagram, Fig. 8). The HRP reaction product was distributed almost exclusively in cells of this restricted 1.1 mm X 0.7 mm region (see photomicrograph, Fig. 9) but a very small number of cells (fewer than 15) in the same nucleus on the ipsilateral side also accumulated HRP reaction-product.

The HRP reaction product in this nucleus took the form of numerous small brown granules distributed within the cytoplasm of the cells. The HRP reaction product in some cells extended into the dendritic processes.

In the nucleus, HRP reaction-product accumulated in multipolar neurons which ranged in size from 9 to 22 μm. The cells of this nucleus were the only cells in the midbrain to become labeled with HRP after intraocular injection. These cells were not as clearly visible in bright field as in dark field microscopy. Also, the intensity of staining varied considerably from cell to cell with respect to the number of granules found.

In the two control turtles injected via the external jugular vein, there was no brown precipitate detected in this nucleus. Blood cells accumulated the reaction-product but they were easily recognizable and did not cause confusion.

Figure 8. Diagram of horizontal section of the midbrain showing the results of injection of HRP into the anterior chamber of the eye. Orientation: anterior toward top of sheet. Central opening: ventricular space. Triangles represent groups of cells which accumulated HRP reaction product and which constitute the restricted nucleus described in the Results.

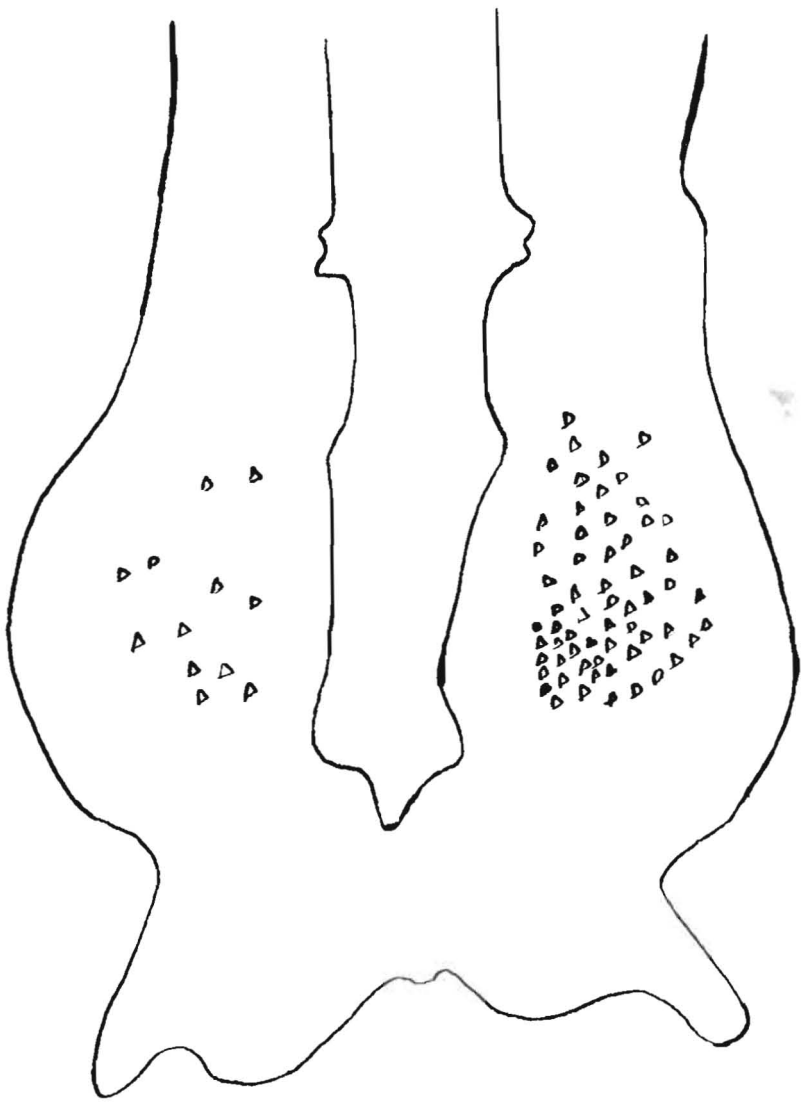
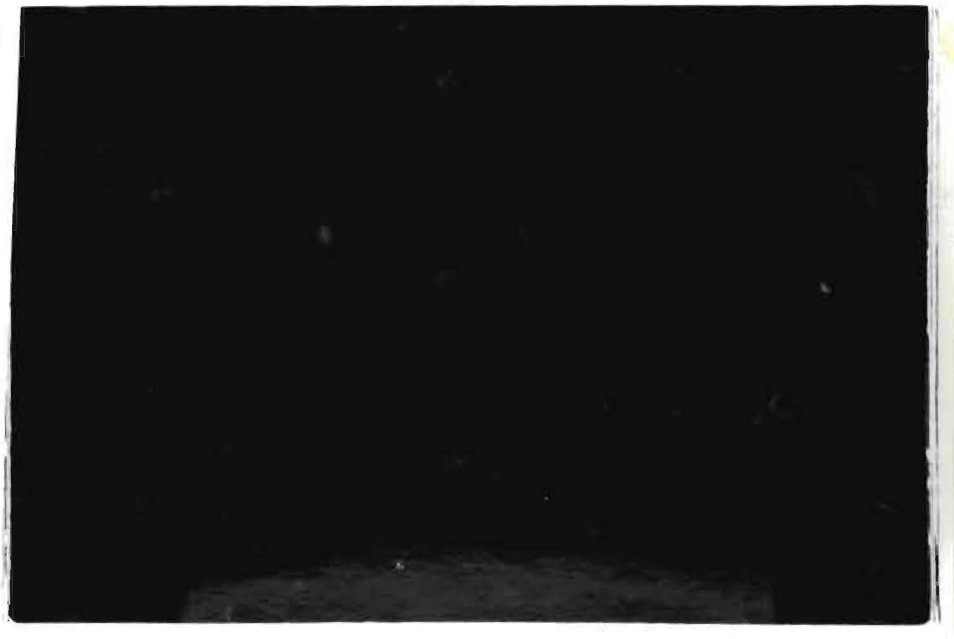


Figure 9. Dark field photomicrograph of a 30 μm horizontal section of the midbrain, showing HRP-labeled cells in the nucleus which is diagrammed in Figure 8. The cells shown were in the side of the midbrain contralateral to the eye which received HRP injection. Magnification 583 X.



DISCUSSION

Evidence for a distributed ganglion along the vagus nerve

HRP injected into the myocardium was transported from endings in the subepicardium and taken up by cells found along the vagus nerves. It is possible, on one hand, that these cells were nerve cell bodies which represent a distributed parasympathetic ganglia. The following characteristics suggest that they were neuron cell bodies: 1) Their sizes and their shapes were similar to those of typical neurons. 2) They had what appear to be axonal processes. 3) The HRP reaction-product had a distribution which is typical for that usually seen in the cytoplasm of labeled neurons, and most importantly it was present in the processes interpreted here to be neurons. On the other hand, the presence of neuron cell bodies along a vertebrate peripheral nerve is unexpected.

This study showed that more of these cells were found along the right vagus nerve after injection of the right ventricle than along the left vagus nerve after injection of the left ventricle. If the cells found in this study are parasympathetic neurons, as suggested here, their larger number in the right vagus would explain why stimulation of the right vagus results in more profound heart inhibition than does stimulation of the left vagus.

The intensity of staining varied from one cell to another, possibly for the following reasons: 1) Some of these cells may have terminals exclusively within the injection site, whereas others may have only part of the terminal field exposed to HRP (Nauta et al., 1974). 2) There could be local variations in the

extent to which HRP is retained in the tissue and to which it remains available for uptake (Nauta et al., 1974).

The blood cells accumulated HRP reaction-product because of their naturally-occurring peroxidases (Welinder, 1976). They were easily differentiated from the nerve cells described here.

Control sections of both vagus nerves showed no HRP reaction-product in the cells identified as nerve cells. Thus, the possibilities were ruled out that the putative nerve cells accumulated HRP reaction-product because of endogenous peroxidase activity or through a vascular route of entry.

Evidence for a midbrain nucleus for efferent fibers to the turtle retina

An efferent control center for the turtle retina could be expected for two reasons: 1) HRP studies have demonstrated efferent nuclei in animals as closely related as snakes (Halpern et al., 1976) and birds (Reiner and Karlen, 1982). 2) Connections by which a central nervous system can directly influence its sensory input at the level of the receptor are known from several systems, a point which will be expanded in the next two paragraphs.

In the auditory system, efferent fibers from brain to cochlea influence the sensitivity of the receptors and may be involved in raising hearing threshold so background noise is ignored (Dewson, 1967). In the proprioceptive system, the gamma efferents to the muscle stretch receptors determine threshold length for firing (Granit, 1955).

Thus in both hearing and proprioceptive systems, the sensory organs are not simple passive recorders relaying information to the brain; instead, the brain can pre-set the sensitivity of its receptors and may be able to shape the sensory input in other ways. In this context it should not be surprising that the visual system would also have efferent connections from the brain to the retina. Anatomical efferents to the avian retina were reported as early as 1889 by Ramon y Cajal, and Cowan (1970) has speculated on their function.

The present results suggest that the turtle retina receives efferent input from a pair of midbrain nuclei, with the major input coming from the contralateral nucleus. This suggestion is based on the following results: 1) When HRP was injected into the right eye, a large number of cells in a restricted region of the left midbrain accumulated HRP reaction-products. 2) A smaller number of cells in the same region of the right midbrain accumulated HRP reaction-product. 3) No other region of the right or left midbrain accumulated HRP reaction-product under these conditions. 4) There was no accumulation of HRP reaction-products in the midbrain following control injections of HRP into the vascular system.

HRP-labeled cells of the nucleus of the ventral supraoptic decussation in the garter snake, Thamnophis, were shown to be a source of retinal efferents (Halpern et al., 1976). The cells of that study appear to be similar in their types, and to some extent in their sizes, to the HRP-labeled cells of the restricted nucleus found in this study in the turtle (Pseudemys scripta). Both are

multipolar neurons and measured from 9 to 24 μm.

The nucleus isthmo-optic (IO) in birds was shown to be a source of retinal efferents (Reiner and Karten, 1982). This nucleus is similar in its position and structure to the isthmic field cells which were shown to be a source of a retinal efferent in Caiman crocodilus (Shortess and Klose, 1975). However, both the nucleus IO and the isthmic field cells are distinctly different in their structure and position from the nucleus of the ventral supraoptic decussation in the garter snake Thamnophis. According to Shortess and Klose, this result may reflect the fact that modern snakes and birds do not share as close an evolutionary derivation as do crocodiles and modern birds. The nucleus discovered in this study seems to be similar to the nucleus of the ventral supraoptic decussation in the garter snake, but a much more extensive study would be needed to establish this point.

It has been suggested (Miles, 1972; Pearlman and Hughes, 1976) that the function of the efferent fibers to the avian retina is to alter the receptive field properties of retinal ganglion cells. Studies on the function of efferent fibers to the reptilian retina would be of great interest but apparently have not yet been started.

SUMMARY

An exogenous protein, horseradish peroxidase (HRP), was used to trace efferent neurons of the heart and retina in the turtle (*Pseudemys scripta*). Small doses of HRP were injected into the subepicardial tissue of the heart and after a 48-hour survival period HRP reaction-product accumulated in what appear to be neuron cell bodies along the vagus nerve. Control HRP injections into the external jugular vein did not label these cells, so a vascular route was ruled out.

After injection of HRP into the anterior chamber of the turtle eye, HRP reaction-product was observed in a group of cells which constitute a restricted nucleus in the midbrain. Most of the cells traced by this technique were in the nucleus on the side of the midbrain contralateral to the injected eye, but a smaller number of cells were traced to the ipsilateral nucleus.

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