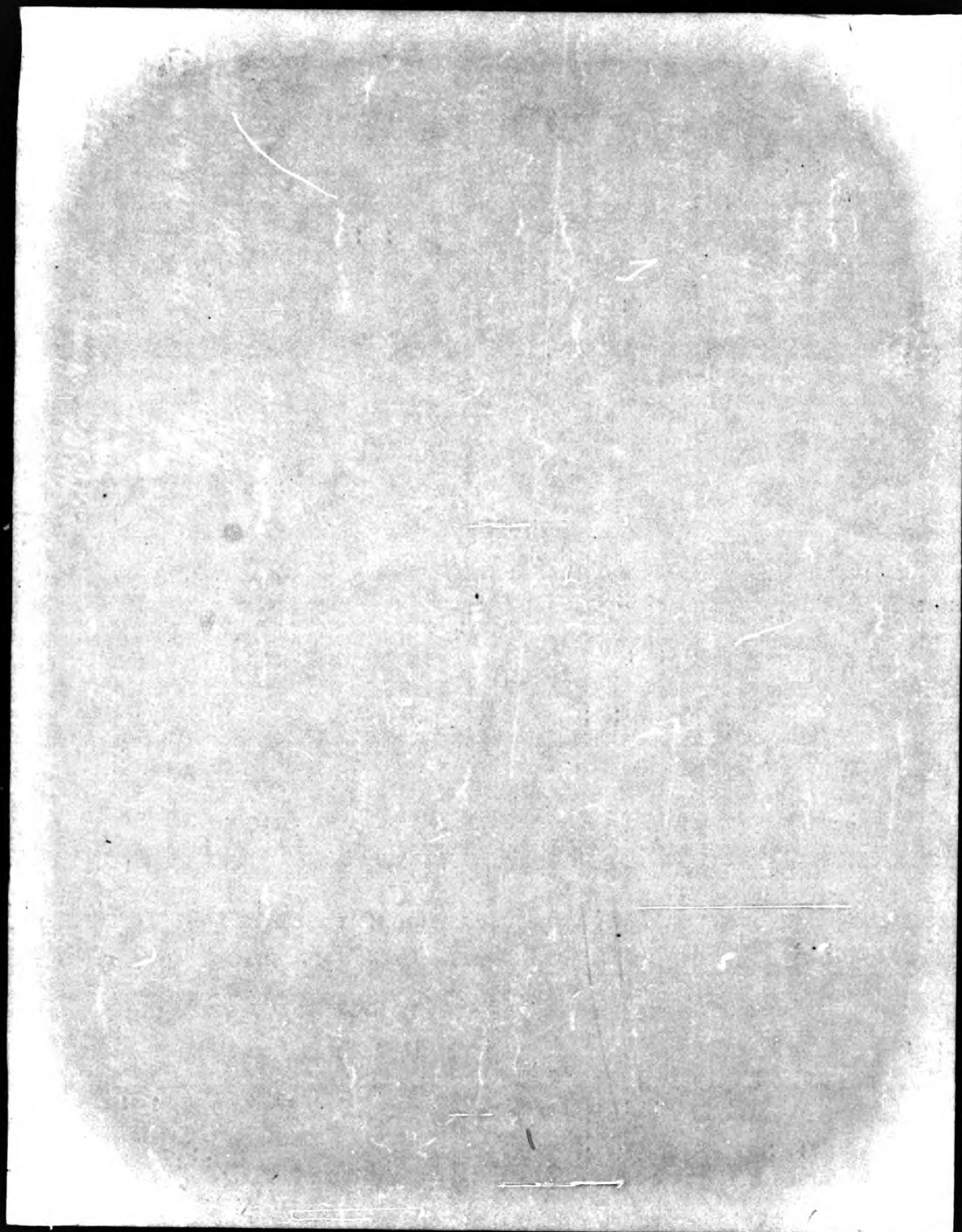

TITLE EXPERIMENTAL STUDIES ON THE ORGANISATION OF THE GUSTATORY SYSTEMS OF THE CARP (CYPRINUS CARPIO)

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EXPERIMENTAL STUDIES ON THE ORGANISATION OF THE GUSTATORY SYSTEMS OF THE CARP, (*CYPRINUS CARPIO*).

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October 1987.

ABSTRACT

EXPERIMENTAL STUDIES ON THE ORGANISATION OF THE GUSTATORY SYSTEMS OF THE CARP (*CYPRINUS CARPIO*).

G.E. BAKER

The enlarged vagal and facial lobes of the carp are associated with the presence of large numbers of taste buds located respectively, within the oropharynx, especially on the palatal organ, and on the body surface, in particular on the barbels.

A literature survey concludes that wheatgerm agglutinin conjugated to horseradish peroxidase, is the most efficient tracer for investigating the central relationships of primary afferent neurons supplying the taste buds. It is shown that this tracer is fifty times more efficient for this purpose than unconjugated horseradish peroxidase.

Central terminals of afferents innervating barbel receptors are shown to be somatotopically organised in the facial lobe and medial funicular nucleus. These structures may therefore incorporate a representation of the body surface which underlies the role of body surface receptors in the localisation of nutrients.

Afferents supplying palatal taste buds course into the vagal lobe only within the deep sensory root and terminate such that a sensory "map" of the palatal surface exists in laminae V and VI. The pattern of distribution of the primary afferent terminals in the vagal lobes resembles the distribution of their terminals in the palatal organ. It is suggested that the vagal sensory map might incorporate a precise representation of the distribution of taste buds in the palatal epithelium.

The motor neurons innervating the palatal musculature are located in the deepest layer of the vagal lobe and in the nucleus ambiguus. The motor innervation is topographically organised in register with the sensory representation.

The representations of the floor of the pharynx and the opposing rostromedial palatal organ are found in similar locations. This organisation probably reflects the integrated activity of both pharyngeal surfaces in sorting edible from inedible constituents of material taken into the mouth.

The separate representations of the internal and external gustatory systems is extended to the central connections of the vagal and facial lobes suggesting that the functional distinction between them is maintained within parallel gustatory pathways.

I hereby declare that this thesis is my own work, except where the contrary is specifically indicated. No other registration for an award of either the CNAA or any University occurred during the period of this research programme.

Advanced studies undertaken with this programme of research included attendance of seminars and conferences.

October 1987

G.K.Baker.

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Finally, my extra special thanks go to Maxine who helped me with many of the figures and managed without my help on many occasions to cope with the needs of our daughter.

CONTENTS

FIGURE PAGE NUMBERS

GENERAL INTRODUCTION

The experimental studies which will be described will be presented in the form of independent chapters, each relating to a component of the gustatory systems of the common European carp, (*Cyprinus carpio*. L.).

Each chapter will have its own individual introduction which will detail the relevant data from previous studies and provide an explanation of the design and specific aims of the studies reported in that chapter. Therefore, in order to avoid needless repetition of the background to each particular study, this initial introduction will simply provide an outline of the topological organisation of the gustatory apparatus in cyprinoid fishes and a description of the gross organisation of the medulla oblongata in cyprinoids. The themes which are common to the studies described will thereby be developed.

Animals receive gustatory information from their environment via taste buds. In air-breathing species, these receptors are found within the oropharyngeal cavity. In some aquatic species, however, taste buds are present both within the oropharynx and on the surface of the body.

The number and regional density of taste buds in

the oropharynx of teleost fishes varies considerably between species, but as shown for example in the catfish, Ictalurus natalis (Atema 1971), the Japanese minnow, Pseudorasbora parva (Kiyohara, Yamashita and Kitoh 1988), and Cyprinus carpio (Herrick 1984), they are predominantly found concentrated on the palate, gill arches and gill rakers.

On the surface of the body, taste buds are often also localised but there is more regional variation. In many species taste buds are found over much of the outer skin but are frequently found more densely distributed in specific locations such as the lips. They are also found in greater densities on morphological specialisations. In the siluroid, Ameiurus melas they are found especially concentrated on barbels located around the buccal opening (Herrick 1981; Atema 1971), and in some gadoids such as the cod, they are present in large numbers on the fins (Herrick 1988).

In teleosts, taste buds are innervated by the facial (VII), glossopharyngeal (IX) or vagus (X) nerve. Of these, the specific origin of the innervation appears to be dependent upon the location of the taste receptor. In general, taste buds on the outer surface of the body, the lips and the anterior regions of the mouth, are innervated by branches of the facial nerve (Herrick 1981). The glossopharyngeal nerve supplies taste buds of the anterior gill arch and the mid-ventral hyoid region (Herrick: in

appendix to Landacre 1907). The vagus supplies the remaining gill arches, the palatal region, the posterior oral cavity and the oesophagus (Herrick 1905).

Using behavioural approaches, the gustatory nature of facial nerve branches innervating the skin was suggested by Herrick (1904) and Parker (1922) and many physiological studies have since supported these observations. In an isolated barbel preparation, taste responses have been recorded from the facial nerve innervation in the catfish, Parasilurus asotus, following the application of various salts (Tateda 1964). Similar chemosensitive units have been recorded from facial nerve branches innervating the skin surface in Cyprinus carpio (Konishi and Zotterman 1961; Funakoshi, Kawakita and Marui 1981).

Although these studies utilised various salts as the gustatory stimuli, some behavioural data indicates that certain amino acids are potent gustatory stimulants. For example, in the pigfish, Orthopristis chrysopterus, the major stimulants of feeding from normal food extracts are substances of less than approximately 1000 molecular weight. Synthetic mixtures of a number of amino acids and the amino acid derivative betaine, have been formulated which could account for the property of the extracts (Carr, Blumenthal and Netherton 1977). Similarly, in the puffer, Fugu pardalis, certain amino acids and betaine have been shown behaviourally to be the major stimulatory

components of extracts of the short-necked clam, which is a constituent of the puffer's diet (Hidaka 1982). In addition, the same study showed, electrophysiologically, a low threshold response to these components in branches of the facial nerve innervating the lips and anterior region of the mouth of the puffer. Studies of the facial taste system in Cyprinus carpio (Marui, Harada and Kashara 1983), Ictalurus punctatus (Davenport and Caprio 1982), Pseudosbora parva (Kiyohara, Yamashita and Harada 1981), and Salmo gairdneri (Marui, Evans, Zielinski and Hara 1983) have also shown a high sensitivity of taste receptors to a number of amino acids.

In addition to the facial taste system, chemosensitive units have been recorded in the glossopharyngeal and vagus nerves of the carp (Konishi and Zotterman 1961) and Ictalurus punctatus (Kanwal and Caprio 1983). Again, some amino acids have been shown to be potent stimulants.

The presence of dense accumulations of taste buds in the pharynx and the posterior region of the mouth of teleost fishes is correlated with particular hypertrophy of the gustatory roots of the glossopharyngeal and vagus nerves. Also, the central nervous locations in which these roots terminate are much enlarged, respectively, into glossopharyngeal and vagal lobes on the medulla oblongata. A similar development of the roots of the facial nerve innervating surface taste buds is present, and is associated with the presence of an enlarged facial lobe,

where these roots terminate (Herrick 1905).

In British cyprinoids, these features are evident and the development of the different medullary lobes has been associated with contrasting feeding behaviours (Evans 1948). For example, in gudgeon and barbel, the dorsal aspect of the medulla oblongata is dominated by the presence of a very large facial lobe. These fish are bottom-feeders and use their well-developed barbels to search the environment for food. In contrast, cyprinoids such as goldfish and bream have much larger vagal lobes relative to the size of the facial lobe. These fish feed predominantly by sucking in the substrate of their environment from which they extract any foodstuffs and eject whatever remains. Evans (1948) suggested that this process of sifting, sorting and retention or rejection of imbibed material was associated with activity of the palatal organ, a prominent mass of tissue on the roof of the mouth.

Similarly, studies of Indian cyprinoids (Bhimachar 1935) and North American cyprinoids (Evans 1952) have subdivided species into "mouth tasters" and "skin/barbel tasters" on the basis of hindbrain morphology.

Thus, in cyprinoids, increased development of the facial gustatory roots and their site of central termination, the facial lobe, appears to reflect a feeding pattern characterised by active searching of the environment using taste receptors located, in general, on

the surface of the animal. Increased development of the vagal roots and vagal lobes appears to be correlated with the presence of dense accumulations of taste buds in the mouth, and especially with the palatal organ which is involved in extracting nutrient material from the various objects taken into the mouth.

A similar functional division of the facial and vagal systems has been observed, behaviourally, in a siluroid catfish, Ictalurus natalis (Atema 1971). Ablation of the facial lobes of this fish resulted in an inability to localise a food source. Ablation of the vagal lobes prevented, not the localisation but, the ability of the animal to ingest food after it has been taken into the mouth. It has been suggested that this may be due to an inability to discriminate between palatable and unpalatable material (Finger 1983).

Cyprinus carpio represents an intermediate form of cyprinoid species in terms of gustatory development. Unlike many of the other cyprinoids, which possess either a highly developed facial or vagal taste system, in this fish both the midline facial lobe and the bilateral vagal lobes are well-developed (Fig.I.1). In view of this, Cyprinus carpio is an ideal animal for investigations of both the facial (or external) and the vagal (or internal) gustatory systems.

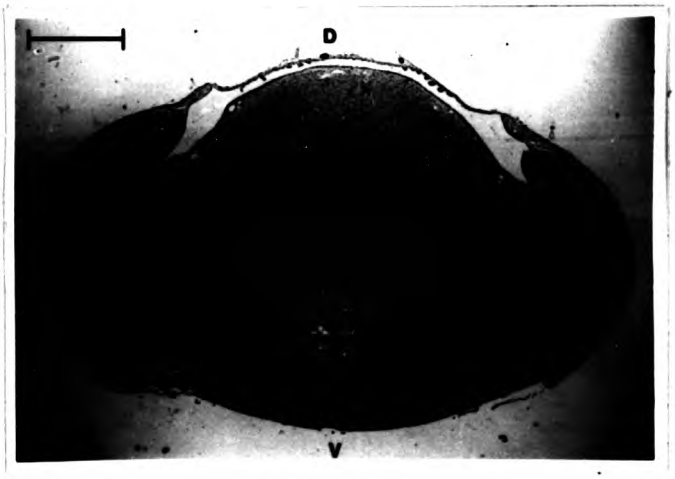
Figure I.1

This figure shows a coronal section at the level of the rostral medulla oblongata. The presence of the bilateral vagal lobes (VL), bilateral glossopharyngeal lobes (GL) and the midline facial lobe (FL) are indicated.
The section has been stained according to the Bodian silver impregnation method (Clark 1978).

D: dorsal. V: ventral.

Scale bar: 1 mm.

Figure I.1



The internal gustatory system:

The oropharyngeal cavity of Cyprinus carpio is functionally divided into three zones as far as the feeding mechanism of this species is concerned. A buccal cavity is present in the forwardmost position. Behind this is found the pharyngeal region divided into anterior and posterior portions. The limits of the anterior pharynx are described by the rostral- and caudalmost extents of the palatal organ (also known as the pharyngeal pad) (Fig.I.2) and (Fig.I.3).

The first four ceratobranchial and epibranchial bones support the gills and gill rakers which form the lateral wall of the anterior pharynx and support the greater part of the palatal organ (Girgis 1952). The posterior division of the pharynx is characterised by the masticatory apparatus i.e. the pharyngeal teeth and the dorsal chewing pad against which the dentition operates. Immediately behind these features lies the oesophageal opening.

The palatal organ (or pharyngeal pad), has been described by a number of comparative anatomists. According to Cole (1944), Aristotle "saw the palatal organ of the carp, which he says is so fleshy that it might be mistaken for a tongue. This interesting sense organ, long regarded as a tongue by fishermen was first described by Rondelet in 1554, and again in 1667, presumably by Swammerdam."

Swammerdam is reported to have written that "that

Figure I.2

This shows the location of the palatal organ (PO) on the roof of the pharynx in a gross dissection. The gill arches (G) can be seen laterally and caudally of the palatal organ. The dotted line indicates the limits of the palatal organ. The dorsal lip (DL) is to the right.
Scale bar: 1 cm.

Figure I.3

This photomicrograph of a coronal section through the cranial region, stained according to the Bodian silver method (Clark 1978) shows the palatal organ (PO), on the roof of the pharynx, and the hyoid protrusion (HP) on the floor of the pharynx.
G: gill arches. D: dorsal. V: ventral.
CNS: central nervous system.
Scale bar: 2.5 mm.

Figure I.2

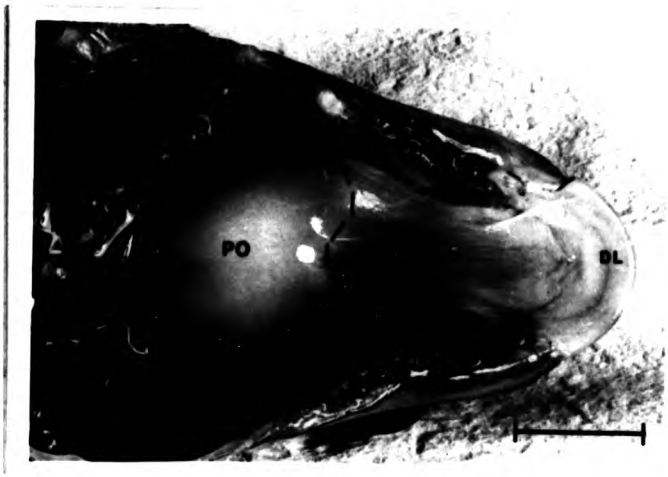
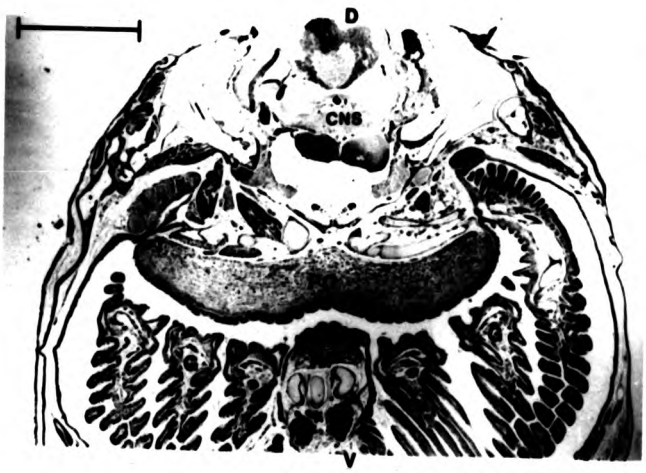


Figure I.3



which is commonly named the tongue is not the true tongue, since it belongs to the upper palate and the food passes underneath it. This so-called tongue consists of a glandular substance, is white, soft and tumid, and when pricked or injured in any way it exhibits rapid movements of an extraordinary character" (Cole 1944). The term "palatal organ" seems to derive from the description of the structure by Valatour in 1861, since when it has been called "Valatour's tongue" or "the palatal organ of Valatour" (Girgis 1952).

Taste buds are present throughout the oropharyngeal cavity but are greatly concentrated in the epithelium of the palatal organ (Fig.I.4), in which they were first described by Weber in 1827 (Herrick 1984). At this epithelial level, in addition to taste buds are found numerous mucous cells.

In the submucosal layers are found matrices of collagen and striated muscle fibres (Matthes 1963) (Fig.I.5). Although, these appear to have no regularly-organised arrangement in the deeper submucosal layers, at the most superficial levels adjacent to the epithelium the striated fibres are generally organised parallel to the palatal surface in two planes orthogonal to each other (Fig.I.6). In addition to this "bed" of muscle fibres, individual striated fibres can be observed traversing the epithelium and associated with the bases of taste buds (Fig.I.7). Within the matrix of muscle fibres are abundant

Figure I.4

Arrangements of cells indicative of the structure of taste buds (TB) can be seen in a transverse section through the palatal epithelium. Many of these taste buds are found on elevated papillae.
Bodian silver method (Clark 1978).
Scale bar: 20μm.

Figure I.5

This photomicrograph of a transverse section through the sub-epithelial levels of the palatal organ shows the matrix of striated muscle fibres (MF).
Bodian silver method (Clark 1978).
Scale bar: 45μm.

Figure I.4

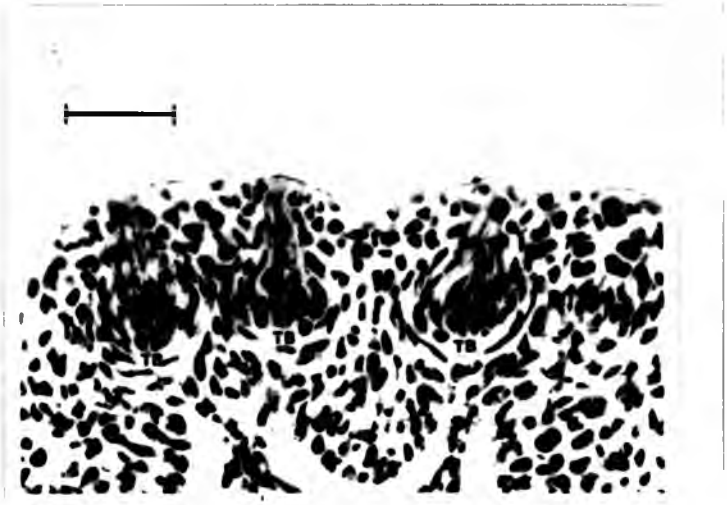


Figure I.5

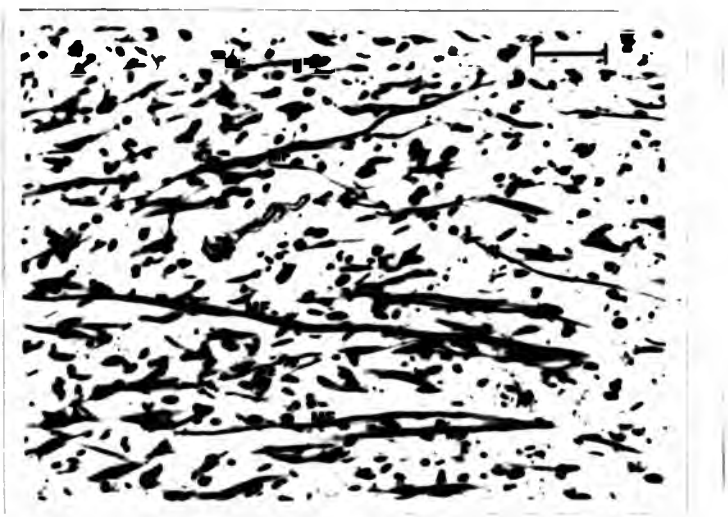


Figure I.6

This is a photomicrograph of a horizontal section through the palatal organ just below the epithelial layer. Populations of striated muscle fibres (MF) at this level can be seen to be arranged roughly orthogonally to each other.
Dark field illumination of unstained tissue.
Scale bar: 50μm.

Figure I.7

This shows a transverse section through the epithelium and subjacent level of the palatal organ. Striated muscle fibres (MF) can be seen traversing the epithelial border into the elevated papilla.
The numbers of muscle fibres generally correlate with the numbers of taste buds (TB) identifiable in each papilla or accumulation of taste buds.
Bodian silver stain (Clark 1978).
Scale bar: 20μm.

Figure I.6

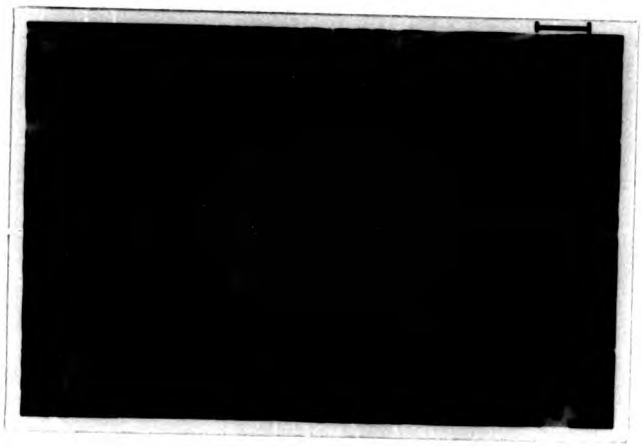
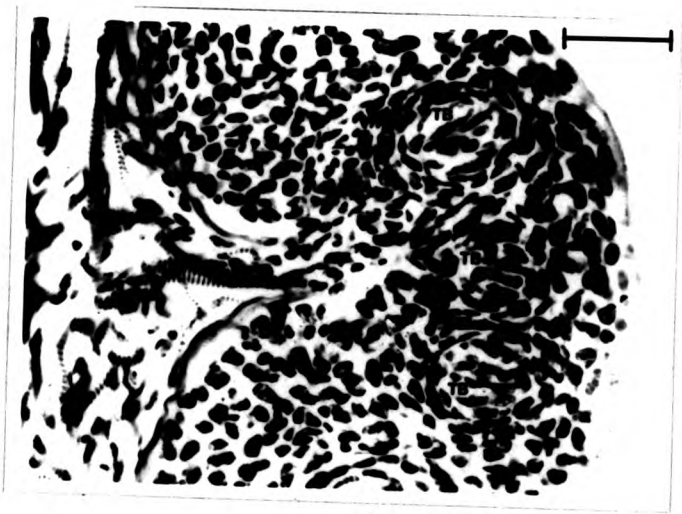


Figure I.7



fat cells and this organisation has been described as providing the palatal organ with an "oleostatic skeleton" (Edgar 1983).

As previously mentioned, speculations on the function(s) of the palatal organ have emphasised a role in sifting and sorting, retention or rejection of nutrient material by virtue of its epithelial taste buds and muscular substratum.

In behavioural experiments using the goldfish, it has been suggested that the palatal organ is active in discriminating between small pieces of material such as heart and plasticine. Because the local protrusion of the surface of the palatal organ, which can be induced by manual probing, is also elicited by natural or electrical stimuli, it was concluded that the muscular response could serve to adjust the position of the ingested particles on the surface of the organ or direct them against the gills and rakers, eventually mediating the discrimination between edible and inedible material (McGlone 1977).

Evidence from experiments which used an X-ray cinematographic technique for visualising the movements of objects in the oropharyngeal cavity of the carp during feeding has supported this view (Sibbing 1982).

In view of its position on the dorsal aspect of the anterior pharynx the palatal organ requires an opposing surface with which to operate in the movement of material.

Its relatively large size brings it close to the base of the anterior pharyngeal cavity and its concave shape closely matches that of the floor with a shallow midline depression running rostrocaudally (Fig.I.3).

In a position opposite the anterior region of the palatal organ, on the floor of the anterior pharyngeal cavity, there is a small structure which resembles the palatal organisation. That is, it possesses taste buds in its epithelial surface and a matrix of underlying striated muscle fibres (personal observation; Fig.I.3; Fig.I.8). By virtue of this organisation, it seems likely that this structure may also be involved in the processes of sifting, sorting and retention or rejection during feeding in the carp.

The palatal organ is innervated by branches of the vagus nerve which project centrally to the vagal lobes. A more detailed description of the structure of these lobes will be given in the appropriate section (Chapter 3). Briefly, it is a laminated structure divided basically into three zones; a superficial, sensory zone, an intermediate fibre layer, and a deep motor region (Herrick 1905; Fig.I.9). The sensory zone receives the primary afferent fibres via two vagal sensory roots. A superficial (or capsular) root separates from the main root and courses over the vagal lobe, whilst the main root enters the sensory zone at a deep level. The motor region

Figure I.8

In a coronal section through the hyoid protrusion, taste buds (TB) can be observed in the epithelium supported by a matrix of striated muscle fibres (MF).

Bodian silver stain (Clark 1978).

D: dorsal. V: ventral.

Scale bar: 200 µm.

Figure I.8

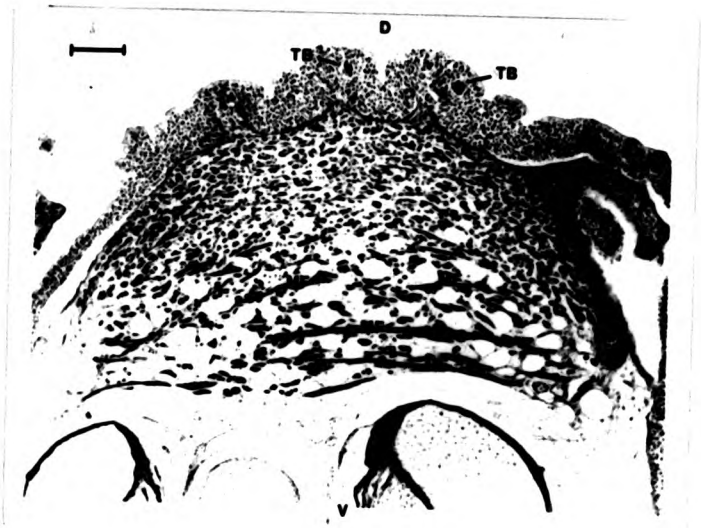


Figure I.9

This horizontal section through a vagal lobe shows the laminar organisation of the lobe. The most superficial layer of the outer sensory zone (S) is the capsular root of the primary afferent input (C).
The fibre layers (F) incorporate the deep primary afferents to the vagal lobe (D) and efferent fibres from the outer layers of the lobe (E).
The inner motor zone (M) constitutes motor neuron perikarya and the motor root from the vagal lobe.
Bodian silver stain (Clark 1978).
Scale bar: 100µm.

Figure I.9

comprises of large perikarya which, according to Herrick (1905), are the motor neurons which innervate the palatal musculature. The intermediate layer constitutes the secondary projection fibres from the vagal lobes.

There appears to be no readily accessible data regarding the muscular structure on the floor of the anterior pharynx. Indeed it has not been possible to find any reference to the the bed of striated muscle fibres at this position in Cyprinus carpio. In fact, Gunther (1880 - cited in Herrick 1904) stated that " the tongue is often entirely absent, and even when it exists in its most distinct state it consists merely of ligamentous or cellular substance, and is never furnished with muscles capable of producing the movements of extension or retraction..."). There is however, reference to a tongue in the Japanese minnow, Pseudorasbora parva, but concentrations of taste buds were only described at the base of the structure (Kiyohara et al 1980).

Taste buds have been described at this location in siluroids (Landacre 1907), but there appear to be no reports of a similarly-positioned pad of muscle fibres. In view of its position on the floor of the anterior pharynx, and for the purpose of reporting the experiments carried out on this structure (Chapter 5), it will be referred to as the "hyoid protrusion".

Little is known about the organisation of the

primary afferent projections from the palatal organ and the hyoid protrusion in cyprinoids. The studies reported in Chapters 3, 5 and 6 were carried out in order to provide more data regarding their central relationships and the pattern of taste bud innervation.

The external gustatory system

The taste buds on the skin of some fishes were first observed by Leydig in 1851 and were further and more accurately described by Schulze in 1863 (Herrick 1984).

The outer surface of Cyprinus carpio is generally studded with taste buds. This fish possesses two bilateral pairs of small barbels associated with the lateral limits of the upper lip. Similar to the lips, these barbels are densely populated with taste buds. In addition, large numbers of taste buds are found on the flanks.

A number of branches of the facial nerve innervate taste buds located around the mouth and over the cranial region. In Ameirus melas (Herrick 1981) and Gadus morrhua (Herrick 1988), as well as Cyprinus carpio (Kiyohara, Shiratani and Yamashita 1985), the major branches are the ramus mandibularis, ramus maxillaris and ramus palatinus. The flank taste buds are innervated by the ramus facialis recurrens which, as also found in the goldfish (Zottoli and van Horne 1983), courses within the posterior lateral line nerve (Kiyohara et al 1985).

In accordance with the classical view, the presence of dense accumulations on the outer surface and especially on the barbels is associated with a well-developed facial lobe which receives the facial nerve input. It may, however, be an overgeneralisation to relate this hypertrophy of the facial lobe in cyprinoids solely to the barbels. It has been shown in catostomid fish that barbels are not necessarily required for the enlargement of facial lobes and that in these fish there is a morphological association between lip development and facial lobe size (Miller and Evans 1965). Admittedly, the lips of these suckers appear greatly enlarged and specialised compared with Cyprinus carpio but it may serve as an indication that the development of the facial lobe in the carp is at least partially correlated with the presence of large numbers of taste buds on the lips.

The facial lobe of Cyprinus carpio, unlike the vagal lobes, does not appear to be a laminated structure, except perhaps for the presence of a distinct external layer (Fig.I.18). The neurons of the facial lobe have been subdivided into large, medium and small cells (Yasgashi 1973) on the basis of soma size, and into six classifications in Carassius carassius mainly on the basis of soma size and dendritic arborization (Morita, Murakami and Ito 1983). According to Herrick (1905), small cells at the centre of the facial lobe in Cyprinus carpio are arranged

Figure I.18

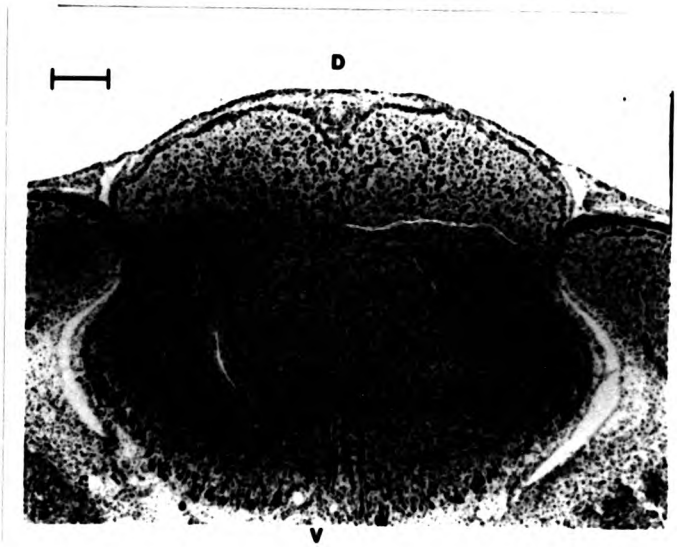
This photomicrograph of a coronal section through the facial lobe (FL) shows that, unlike the vagal lobes, the structure is not laminated except for a clearly definable outer layer. Stained fibres can be seen at the ventral extent of the lobe (F). These are probably myelinated fibres which constitute the primary afferents to the facial lobe.

Bodian silver stain (Clark 1978).

D: dorsal. V: ventral.

Scale bar: 150μm.

Figure I.18



in dense clusters or rosettes. It has been speculated that small cells receive the primary afferent input to the facial lobe in this species and are intrinsic to the lobe (Yasgashi 1973). It might be argued that this gains support from retrograde tracer studies in the bullhead catfish (Finger 1978) and crucian carp (Morita et al 1983) in which small cells remain unlabelled following horseradish peroxidase injections into locations within the brain which are known to receive the facial lobe efferent projections.

Studies of the primary afferent projection to the facial lobes in Ictalurus have shown that a gustatory somatotopy is present across the lobes (Finger 1976). In Cyprinus carpio, electrophysiological studies of taste responses in the facial lobe have suggested that a similar somatotopy exists (Marui 1977). Also, studies of tactile responses in the goldfish facial lobe have suggested a topographical organisation (Peterson 1972). The experiments on the external gustatory system described in Chapter 4 were carried out in order to investigate this further using a sensitive neuroanatomical tract tracing technique.

Central gustatory pathways.

The pattern of neuronal connections with the medulla oblongata appears to be basically similar across

many teleost species. However, in fish possessing elaborate gustatory systems, not only are the vagal and facial lobes well-developed relative to their structure in microgustotic species but, the secondary projections originating from these primary relay sites are also relatively hypertrophied.

Most of the relevant data has been reviewed by Finger (1983). Briefly, an ascending secondary gustatory tract arising from the vagal and facial lobes courses rostrally to terminate in the pontine superior secondary gustatory nucleus. This nucleus is enlarged in "gustatory" species (Fig.I.11). In Ictalurus, some secondary fibres project to the nucleus lobobulbaris in the inferior lobes (Finger 1978). This latter projection, however, appears to be absent in Carassius carassius (Morita, Ito and Masai 1980; Morita et al 1983). A descending secondary tract terminates in regions at rostral levels of the spinal cord.

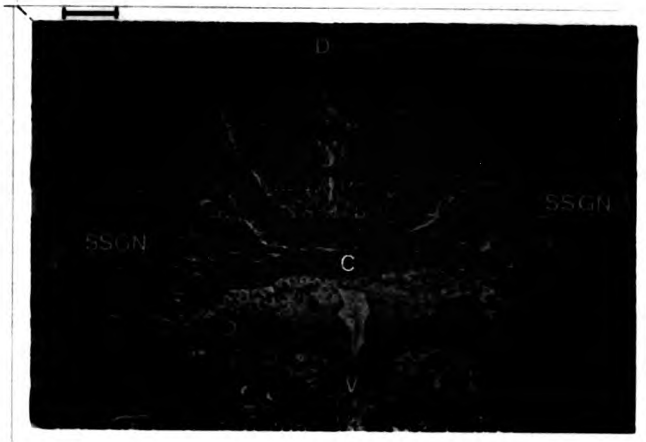
In addition to the primary afferent input, the vagal and facial lobes receive afferents from central structures. The major sources of these inputs arise in the inferior lobes.

The experiments described in Chapter 7 were designed to demonstrate the central connections of the vagal and facial lobes in Cyprinus carpio. These may then be compared with those observed in other

Figure I.11

This shows a coronal section at a pontine level indicating the presence of the large superior secondary gustatory nucleus on each side (SSGN) and the commissure (C) coursing between the nuclei.
Holmes silver stain (The negative for this print was kindly lent to me by Dr.M.G.Roberts).
D: dorsal. V: ventral.
Scale bar: 200μm.

Figure I.11



species, especially in relation to differences between the species as regards the development of the peripheral gustatory apparatus.

Methodological considerations.

Many of the experiments described utilise a recently developed axonal tracer, wheatgerm agglutinin covalently conjugated to horseradish peroxidase. Coupled with a sensitive method for the detection of the horseradish peroxidase component of the conjugate, the use of this method has been found to be particularly useful for tracing efferents and afferents of many neuronal pathways, especially in mammals. However, it does not seem to have been widely used in studies of lower vertebrates. Also, it might be argued that a number of the investigations reported here could have yielded similar data using more established methods. In view of these factors, various features of neuroanatomical tract tracing have been reviewed in Chapter 1 with a view to explaining the rationale for using the particular methods employed in the studies of the gustatory systems of Cyprinus carpio.

In addition, a number of the studies have provided data on the dynamics of uptake and axonal transport of wheatgerm agglutinin conjugated to horseradish peroxidase. Consequently, the studies described will include some qualitative and quantitative assessment of the advantage of using this tracer for particular investigations of

lower vertebrates.

CHAPTER 1

TRACT TRACING METHODOLOGY.

At this stage, prior to presentation and discussion of the investigations carried out on the cyprinoid gustatory systems, it is appropriate to present an examination of the various approaches to answering the questions asked by the experimental neuroanatomist.

In view of the multiformity of schemes available, including those that have been and those that are currently popular, the goal of this exercise is to provide a methodological framework within which the techniques used in the succeeding experiments can be seen to have developed.

As the greater proportion of the tract tracing procedures followed during these investigations of the gustatory projections have utilised the histochemical detection of the enzyme horseradish peroxidase (HRP), this composition will be mainly concerned with the evolution and development of this technique and its variants. In addition, the advantages and limitations of the methods used can be compared with the use of, or combination with, possible alternative strategies.

In order to comprehend neurobiological systems and their ethological significance it is clearly important to understand their organisation. The conjunction of this

knowledge with the relevant physiological data can provide an intimate view of the operating matrix and its functional units.

From an anatomical standpoint, the pioneering investigators of neural structures were dependent upon the analysis of nuclear and fibre morphology in normal tissue. That is, tissue which had not previously been, directly or indirectly, experimentally manipulated. Oblique inferences were possible though, from comparisons between pathological specimens and those from non-diseased sources. For example, patients with Parkinson's disease were found to have undergone cell loss in the substantia nigra compared with unaffected individuals, and thus affording a path-finding initiative in the search for the central nervous malfunction(s) responsible fo this motor disorder (Brodal 1969).

The visualisation of neural structures was made possible by the development of dyes which varied in their affinity for various tissue constituents.

The foundations of microscopic anatomy had, however been laid prior to the advent of staining techniques. Axons and their sheaths had been identified by Remak in 1836 and the output neurons of the cerebellum were similarly described by Purkinje, through the observation of unstained sections (Williams & Warwick 1975).

Nevertheless, neurocytological analysis and the associated theories of nervous system structure made a

"quantal leap" due to the availability of pigments which provided enhanced visual contrast of selected cell components.

These early stains were extracted from the flora and fauna, for instance, haemstoxylin from the bark of the tree Haematoxylon campechianum and carmine from the insect Dactylopius cacti (Carleton & Leach 1938), and thus allowed a window, albeit frosted, to open onto the details of neural microstructure.

The first use of such a stain for neurocytological purpose has been attributed to Gerlach (1858 - in Drury & Wallington 1967) who differentiated cell bodies and fibres in a cerebellar section. An important advance was also made using a natural extract by Dieters in 1865 who distinguished between two fibre types originating from a motor neuron cell body.

Staining methods using these natural pigments were however superseded due to the manufacture of dyestuffs, particularly for the textile industry, and their subsequent beneficial use for improved histological method.

These synthetic products were based on the aniline derivative of the benzene ring and the impact of their formulation can be gauged in the continued use of a number of them, e.g. toluidine, thionin and cresyl fast violet, in routine procedures for CNS cytological observation.

The usefulness of these particular stains lie in their binding affinity for Nissl substance: hence the

generic term, Nissl stains. Although this material was presumed to be an artefact of fixation, the implication of a groundless existence was confuted by finding it observable in nerve cells but not other tissue, and its decrease or disappearance (chromatolysis) in damaged cells. It is evident now that the sites of "binding" of these stains are those which contain quantities of nucleic acid and the cellular region most densely stained is thus, generally, the cell body. The method is consequently particularly suited to analysis of nuclear groups.

The use of these stains, in conjunction with others which identify other cell components such as neurofibrils, provided fertile means for accumulating cytological data i.e. that concerning, in the main, structures "within" the cell. However, by their nature, these means afforded little detail of the microanatomy of the tissue i.e. the relationships "between" cells and their processes. It is not surprising in retrospect, therefore, that the "reticular theory" of nervous organisation prevailed, whereby the processes of each cell were envisaged as continuous with those of neighbouring cells.

The development of a different class of techniques, effecting impregnation of various tissue features with heavy metallic derivatives, enabled a more thorough description of intrinsic and extrinsic associations i.e. respectively, between neurons of a group, and between groups of neurons.

Unlike the relatively straightforward staining of tissue components with coloured dyes, these methods relied on precipitation of a metallic salt upon the cell or cellular constituent. The opaque deposit thus produced created a visual contrast between the impregnated cell and its background, consequently allowing microscopical pursuit of the neural processes.

Since Cajal (1903) and Bielschowsky (1902) (in Palmgren 1948) introduced their silver nitrate methods for demonstrating axons, a variety of reduced-silver impregnation procedures were developed (and are still being modified e.g. Cruz, Jeanmonod, Meier & Van der Loos 1984) to suit different preparations. However, a method which is optimal for demonstrating branches of processes may have the disadvantage of not depicting other features, such as large axonal bundles. Additionally, peripheral and central nervous tissues may have different susceptibilities to the same method.

Thus, a number of variants have been developed and remain widely-deployed for normal tissue, notably the silver-proteinate protocol of Bodian (1936). The methods of Holmes (1943) and Palmgren (1948) (especially useful for peripheral nerves and endings) were introduced because of the transient unavailability of "protargol" silver-proteinate. A technique developed by Marsland, Glees & Erikson (1954) has also been extensively used.

A significant addition to the technical armoury of

the neuroanatomist was provided by the development of methods using heavy metal impregnation (silver or mercury) of tissue which had previously or concommitantly been fixed with chromate and/or dichromate salts.

The efficacy of this combination was discovered by Golgi in 1873 but became universally known through the works of Ramon y Cajal who applied it to many preparations of nervous tissue.

The proliferation of variants of the basic procedure led to their general categorisation as the "Golgi methods", but their unifying feature and enormous advantage was the impregnation of only a small proportion of the densely-packed elements. This, therefore, enabled scrutiny of the dendritic expanse of individual neurons and the course of their axons, characteristics which had previously been largely precluded by confusion within the background. The interpretation of Cajal, that the impregnated feature was the nerve cell in its entirety, gave considerable weight to, and led to widespread acceptance of, the "neuron doctrine" of nervous system structure.

The initial decades of this century saw a profusion of studies using the benefits of the Golgi methods to analyse the connections of distinct neuronal populations and the morphologies of the constituent cells.

The effectiveness of these particular strategies can, again, be best adjudged by their continued use in quantitative and qualitative neurobiology and the frequent

verification of Golgi-derived observations using more recent, sophisticated procedures.

Concern, so far, has been directed at the neuron itself: the visualisation of the cell body and the route(s) of its associated processes, the dendrites and axon. Axons, however, and especially those which project to relatively distant zones, are frequently ensheathed by myelin either singly or in bundles.

As the physico-chemical nature of myelin is quite different from that of the process(es) it envelops, various methods and modifications were developed to selectively stain this material in tissue sections (e.g. Weigert 1884, Pal 1886, Kultschitsky 1898: in Drury & Wallington 1967). Thus the course of myelinated fibres could be followed by tracking the stained ensheathment through adjacent sections.

Following specifically-stained structures through adjacent sections however, could present problems when the tract of interest merged with or coursed closely to another tract. Similar staining of the two tracts could give rise to much confusion. In addition, a projection involving relatively few neurons could be easily lost within the surrounding neuropil. Clearly, it was necessary to experimentally define the neurons under investigation differentially from others.

Experimental degeneration

Crucial advances had been initiated in this direction from the use of "reactive" changes in neural tissue following damage. As early as 1850, Waller had demonstrated observable changes in the distal portions of transected nerve fibres and this disintegration was subsequently referred to as Wallerian or anterograde degeneration (Bowsher, Brodal & Walberg 1968). This deterioration also affects the myelin sheath and the observation that degenerating myelin could be selectively impregnated with osmic acid after pretreatment with chromium salts whilst normal sheaths remained undarkened, provided Marchi & Algeri 1885 with a technique for tracing myelinated projections from a known point of lesion (Brodal 1969).

In some circumstances, atrophy of the proximal part of the axon had also been observed (von Monakow 1889a: in Brodal 1969) and this so-called "retrograde" degeneration could also be visualised using the "Marchi methods" which had evolved.

Nevertheless, despite pioneering the use of reactive changes in the nerve fibre for tract-tracing purposes, these techniques for staining disintegrating myelin sheaths were eventually superceded for several reasons.

Firstly, the timing between tissue ablation and the consequent degeneration was critical, varying between

different neuron types and from species to species. Secondly, many fibres are poorly myelinated or unmyelinated and, thus, the effectiveness for tracing their pathways is limited. Thirdly, some confusion could arise because of the lack of myelination of axonal terminals and the possibility, therefore, of misinterpretation of their destination.

The eventual consequence of the drawbacks associated with methods based on degenerative myelin was a change of focus, attention being directed towards degeneration of the axoplasm itself. This approach overcame several of the obstacles to accurate interpretation of lesion-induced damage.

Primarily, it was evident that all affected fibres, whether heavily myelinated or otherwise, could be traced utilising this scheme. Additionally, in theory at least, terminal boutons could be precisely localised due to their disintegration.

The capacity for identifying anterograde axoplasmic degeneration with silver impregnation was expounded, firstly, in the 1930's (Hoff 1932) and subsequently capitalised upon by other investigators (Glees & Clark 1941; Glees 1946) searching for means of examining distant neural connections.

These techniques further established an experimental paradigm, instituted by the Marchi methods, which has

formed the practical basis of a large number of subsequent tract-tracing methodologies. This standard pattern can be regarded as involving five subsets of procedures: surgery, survival, sacrifice, fixation, and detection.

Following the initial experimental "manipulation" of the area under investigation, a period of survival is necessary whereby the metabolic processes of the tissue may proceed sufficiently to optimise the detection of the consequences of the operative strategy. After sacrifice of the animal, specific means of tissue fixation are required according to the particular subject of the detection process.

For example, in the case of the method of Glees (1946) for detecting degenerating terminal boutons, the lesion, induced by knife-cut, aspiration or some other means, was followed by a post-operative period of survival. The extent of the time-period necessary for degenerative mechanisms to progress enough for subsequent optimal detection had to be determined empirically. Thus, a range of survival times would be assessed for prime exposure of the degenerating structures, the optimum being dependent upon the species of animal under investigation, the distance from the lesion to the terminal axonal arborizations, and the body temperature. The optima could therefore possibly vary from several days to several weeks.

Sacrifice and fixation usually involved vascular

perfusion of the anaesthetised animal with isotonic saline, followed by the fixative solution. The brain was then removed from the cranium and stored in the fixative for several weeks prior to sectioning. Detection of the degenerating terminals was then accomplished by exposing the tissue sections to the relevant silver impregnation media.

The Glees method, then, represented the first extensively-used means for visualising a particular projection differentially from the surrounding neuropil Degenerating neurons could be traced to their field of projection and the efferent connections from the lesioned zone could be located.

This particular technique, nevertheless, presented a number of problems for investigators. It was inconsistent, the identity of the deteriorating structure was ambiguous and, most of all, it was a difficult and laborious task to distinguish normal elements from those undergoing degeneration because both types were impregnated, albeit differentially.

A definite requirement was a procedure which impregnated degenerating axons and endings alone against a more benevolently contrasted background.

The development of this capability (Nauta 1950, 1957; Nauta & Gygax 1951,1954; Nauta & Ryan 1952) initiated what can probably be best described as a neuroanatomical revolution due to its provision of a wealth of

significant experimental data about neural projections and the theories of development and organisation this reliable and reproducible information precipitated.

The fundamental concepts underlying the "Nauta methods" were twofold: 1) exploitation of the preferential availability to ammoniacal silver salts (argyrophilia) apparently made possible by the degenerative process itself, and, 2) suppression of the normal fibre impregnation.

The Nauta class of technique was found to be particularly effective for mapping degenerating axons but, disadvantageously in many cases, not especially suitable for localising similarly affected terminals. This limitation was overcome by the evolution of a method which was capable of providing a more substantial impregnation of the entire projection (Fink & Heimer 1967) and which has possibly been the most widely-used of the silver impregnation methods available for identifying degenerating pathways.

Notwithstanding the previous statement, varying success rates tend to accompany the use of a particular methodology in different laboratories with a resultant increase in the number of modifications. This seems to have been nowhere truer than in the case of impregnation of degenerative neural elements, a whole host of "official" variants having evolved to suit different preparations (e.g. Chambers, Chung-Yu & Chan-Nao 1956; de

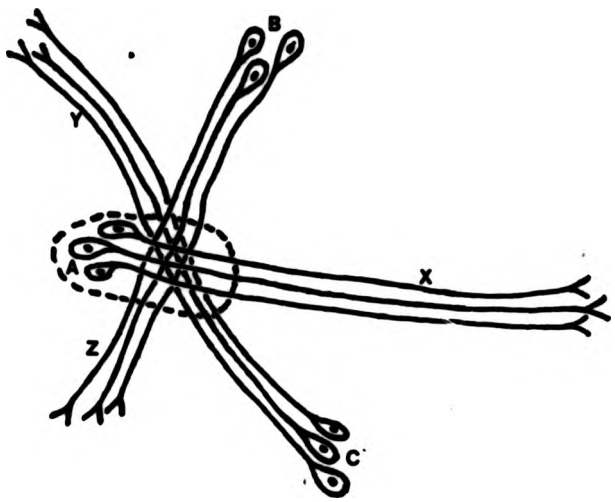
Olmos 1969; Wiitanen 1969; Kager 1970; Hjorth-Simonsen 1970; de Olmos & Ingram 1972; Desclin & Escubi 1975).

So, lesioning or transection of nerve fibres is generally followed by degeneration and ultimate disappearance of the whole distal portion of the injured elements wherever they may project to, and this Wallerian degeneration can be exposed optimally using suppressive silver impregnation methods. The distribution of efferent connections from the lesioned area can thus be traced.

The interpretation of this data however can be ambiguous because the operative technique may have, by its very nature, indiscriminately affected not only the efferents, but also neuronal processes derived from some other source simply passing through or close to the lesioned area (Fig.1.1).

The possibility of falsely positive data cannot be overlooked and this difficulty, the "fibre-of-passage" problem, arises in many tract tracing procedures dependent upon an initial surgical intrusion into the region under investigation. This issue will be raised again and its details thoroughly discussed below in relation to the use of tracer substances.

Figure 1.1



This diagram illustrates the possibility of damaging fibre projections other than those constituting the system under investigation.

Assuming that the projection of interest is that originating at source A, a lesion of this region (circled area) would also include efferents from sources B and C. The lesion is therefore likely to result in degenerative profiles along the routes X, Y and Z thus giving a false impression of the projection field from source A.

A second interpretive difficulty arises out of retrograde cellular reaction i.e. in the proximal portion of the injured fibre(s).

For some time after the observation of the anterograde response it was believed that no similar retrograde process occurred. Even after the initial demonstration of a retrograde response by Cajal it was suggested that the origin of the axon collateral nearest to the lesion was the limit of reaction (Grant 1978). However, as already noted, a chromatolytic change in the soma of damaged cells had previously been described and it is now established that this acute reaction is often followed by complete atrophy of the proximal segment of the neuron. Ranson (1986) had observed loss of cells in spinal ganglia after peripheral nerve transection, and similar retrograde degeneration has been demonstrated in hypoglossal motor neurons (Grant & Aldskogius 1967).

On the other hand, damage to the fibre does not always result in this chronic reaction. It may, instead, be followed by gradual recovery or no alteration whatsoever in the proximal portion, even though the distal segment degenerates. The idea has developed that the variation in the response of neurons to damage may reflect the degree of damage inflicted.

For example, an individual neuron may innervate a particular region and collateralise widely in that area of projection. A small lesion is likely to injure only a

fraction of the processes whereas more and more extensive ablation would damage progressively larger proportions of the axon and its collateral field, with the increasing likelihood of injuring the neuron irreversibly (Fig.1.2).

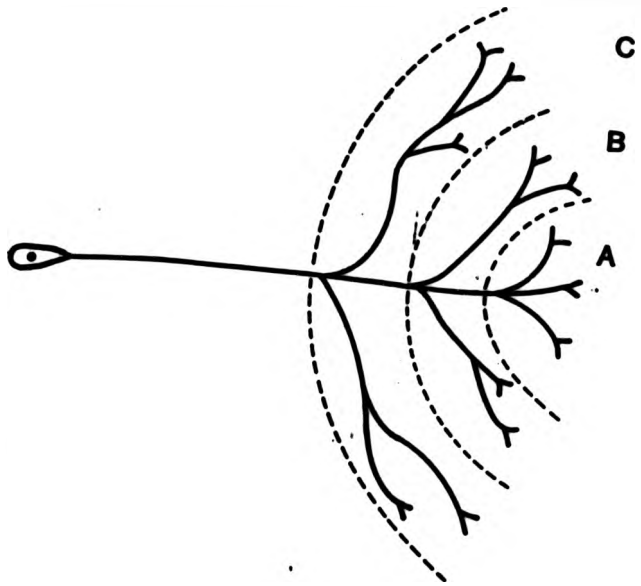
Indeed, it has been suggested that the increase in the number of surviving cells following progressively rostral lesions of the dorsal spino-cerebellar tract in mammals is due to the contribution of greater numbers of uninjured collaterals (Liu 1955).

Additionally, enlarging the extent of a cortical lesion can augment the amount and intensity of retrograde degeneration in the thalamo-cortical pathways (Von Monakow 1889:in Beresford 1966). Thus, it would seem plausible to expect that a degenerative cell body has had the greater proportion of its collaterals and/or its main branch severed. This schema, however, does not explain why some retinal ganglion cells can survive transection of the optic nerve (Madison, Moore & Sidman 1984).

Briefly then, silver impregnation techniques are of great value for identification of degenerating neural elements but translation of data can be prone to false positive and false negative conclusions.

The degenerative process has also been used for investigation of central connections of neurons innervating peripheral structures. As previously exemplified, the retrograde mechanism can be utilised to elucidate the

Figure 1.2



This diagram illustrates the possibility of ablating larger numbers of an axon's collaterals by increasing the size of the lesion.

The small lesion A is likely to damage less of the collateral field of the neuron than progressively larger lesions B and C.

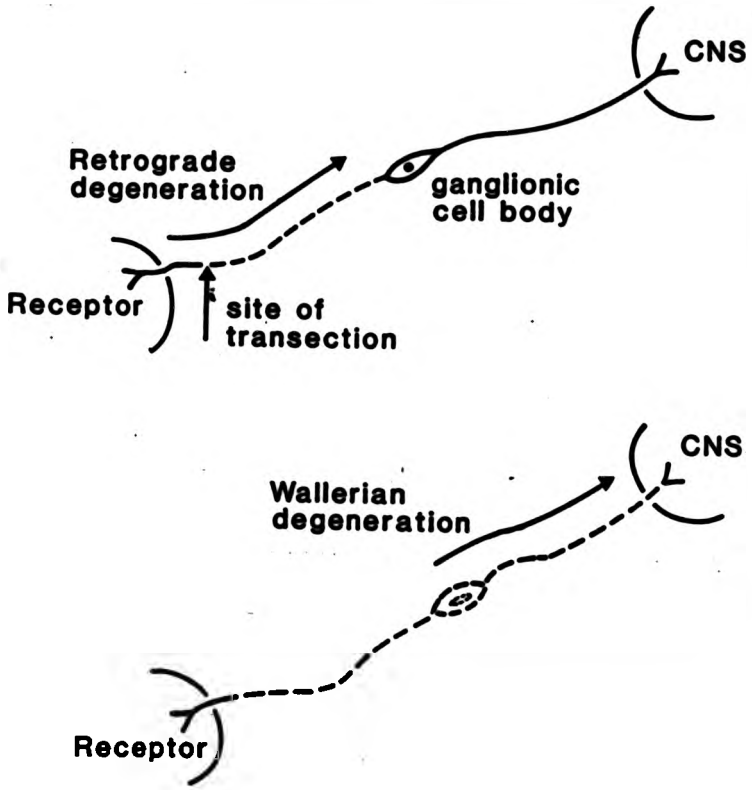
motor neuron source, but the primary sensory neuron clearly represents a less straightforward arrangement.

Early Marchi studies seeded the notion that central degeneration of myelin occurs subsequent to transection of the primary afferent's peripheral branch, but this left open whether an axonal degeneration also takes place.

Using a Fink-Heimer modification, an initial retrograde degeneration has been observed in a trigeminal ganglion followed by Wallerian degeneration in the centrally-directed root, thus indicating that transganglionic degeneration does occur (Grant & Arvidsson 1975) (Fig.1.3).

Despite this success, use of such transganglionic atrophy as a neuroanatomical tool for evaluating primary afferent central distribution does not seem to have been widely favoured. This may be due to, what appear at first sight, quite broad species differences, the amount of transganglionic degenerative debris being much greater in the rat than in the cat. Other features, however, such as postoperative survival times, developmental stage and distance of transection from ganglion would also be expected to be important factors.

Figure 1.3



The upper diagram shows the initial retrograde degenerative reaction following transection of the distal part of a primary afferent neuron.
The lower diagram shows the subsequent anterograde or Wallerian degeneration of the proximal part of the neuron.

Summarising, the introduction of tract-tracing methods based on the mapping of degenerating neurons provided a means for observing the projection of interest independently of the neighbouring tissue. Their use has generated a great deal of data regarding neuronal connectivity.

However, the lesioning procedures required do not discriminate between the specific neurons being investigated and those passing through the disrupted site.

In addition, the methods relied on atrophic changes in the relevant pathways. Therefore, by their very nature, these methods precluded simultaneous investigation of the normal anatomy of the neurons comprising the projection.

Tracers

In contrast to the silver impregnation of degenerating elements, which relies on mapping "pathological" changes in neuronal pathways, the majority of currently-favoured techniques, involving introduction of extraneous tracer substances, take advantage of the normal "physiological" functions of the neurons that constitute the pathways.

The initiative for this approach stems from the studies of Weiss and collaborators who were the first to observe the phenomenon of axoplasmic movement following axon ligation (Weiss & Hiscoe 1948) and subsequently described the active nature of the underlying transport

mechanisms (Weiss 1978).

The characteristic classification of axonal transport is accomplished in terms of its direction and the velocity of its components. As regards the direction, movement "away" from the cell body originally followed the terminology of the degeneration studies - "anterograde"; but the alternatives,"orthograde" and "somatofugal" are frequently used. Similarly, "retrograde" has been retained as descriptive of movement "towards" the perikaryon but is sometimes substituted by "somatopetal".

Concerning the rate of movement, transport in the orthograde direction is generally divided into two broad categories, fast and slow. It has been suggested that further subgroups of these exist (Grafstein & Forman 1980), but the basic features, fast and slow orthograde, and retrograde transport will suffice for explanation of tracer movements.

The concept of applying extrinsic substances for tract tracing was prompted by experiments on the rates, direction and identity of axoplasmic movement in optic nerve fibres (Taylor & Weiss 1965). The use of radioactive protein in their study suggested a rationale for tracing fibre projections with radiolabelled amino acids. Following internalisation of the labelled compound into the neuron it would ultimately distribute intracellularly and be detectable by autoradiography. The distribution would thus be mapped, using the natural processes of the

cell.

The neuron is a metabolically active and functionally differentiated unit. A major proportion of its protein synthesis takes place on ribosomes in the perikaryon with little or none observed in axonal processes. Application of a radiolabelled amino acid to a particular region of tissue would lead to uptake and incorporation into protein by cell bodies in that area. With sufficient post-injection survival, the labelled protein would be detectable in the various neuronal processes. This approach therefore represents a very practical means to tracing efferent projections of the injected zone.

The occurrence of a fast and a slow rate of transport allows observation of different features of the neuron. The materials transported in the fast phase appear to be predominantly particulate (e.g. vesicles and mitochondria) and have been implicated in synaptic function. This component has therefore been used to map the efferent terminals and, because of its rapid velocity, has allowed observation of the terminals without need for precise evaluation of survival time. The slow phase, however, has been related mainly to tubulin, actin and myosin which are concerned mostly with axonal structure and function. Labelling these features, then, provides for demonstration of axonal and collateral trajectories but, being a less rapid process, requires a more calculated approach to survival periods.

The autoradiographical means of detection of the labelled structures puts a number of restraints on the technique. The detection method is second-order, i.e. the primary event is the radioactive decay of the labelled protein in the tissue and the secondary event is the production of a silver grain in a photographic emulsion overlaying the tissue section. It is therefore important to use β-emitting istopes as labels because of their low energy and consequent greater resolution (i.e. less distance between decay and resultant grain). Resolution will also clearly depend on the thicknesses of the section and the emulsion layer. Tritium (^3H) is a low energy β-emitter and is the radiolabel most commonly used for tagging tract-tracing amino acids.

As regards the specificity of amino acids, leucine and lysine were the earlier choices due to their greater natural occurrence in axoplasmic proteins. However, tritiated proline is now the frequent choice due to its greater incorporation into the rapid phase of transport and subsequent heavier terminal labelling after short survival times; tritiated leucine has been found to label predominantly the slow phase (Hendrickson 1982).

Proline has also been found to be less prone to non-specific uptake via the circulation or CSF than leucine, thus decreasing the level of confusing background labelling in the emulsion. Various environmental factors such as cosmic and gamma radiation, and light and thermal

energy, are equally capable of increasing this background labelling. Also, direct reduction of the emulsion's silver halide by chemographical reagents in the sections can be another source of misinterpretation.

In the case of a dense projection these background levels may be low in comparison to the genuine label. However, should the efferents of interest be sparsely distributed, accurate interpretation could be compromised. Although such a diffuse pathway can be "amplified" by extending the time of exposure of the emulsion to the labelled section (the number of silver grains formed is proportional to exposure time), this additionally enhances the opportunities for background reactions to occur.

Orthograde axoplasmic transport autoradiographic tracing (OAAT) was used initially for peripherally-located neurons (e.g.Lasek, Joseph & Whitlock 1968) but was soon found of practical application in central structures (Cowan, Gottlieb, Hendrickson, Price & Woolsey 1972). This work (using tritiated leucine), applied OAAT to neural systems whose distribution was well established and affirmed the pattern of connections demonstrated by the earlier techniques. The method has subsequently become a fundamental part of the neuroanatomical armoury.

This procedure has consistently been used to answer the question "What are the efferent connections of the injected region?". However, implicit in this is the assumption that the labelled tracer has been transported

entirely somatofugally.

Evidence from several sources contradicting this has described substantial retrograde transport of ^{3}H-proline (Kunzle 1977; Reperant, Vasselkin, Ermakova, Kanigfest & Kosareva 1983). Also, use of the neurotoxin kainic acid to destroy cell bodies in the area under investigation prior to ^{3}H-proline delivery (and therefore prevent orthograde transport in the efferents from that area) has demonstrated labelled tracer in cell bodies of known afferents to the injected structure (Levay & Sherk 1983). It seems that the involvement of retrograde transport in OAAT cannot be discounted.

Equally, as for degeneration studies, transneuronal actions cannot be underestimated in ARG data. A number of examples of this transynaptic transfer of labelled amino acids exist (presumably in the form of labelled protein - Reinis & Goldman 1984). Globus, Lux & Schubert 1968 first described the passage of radiolabel from nerve to muscle in the cat, and in central neurons, transneuronal transfer of radioactivity has often been used for tract tracing in the visual system of a variety of mammals (Grafstein 1971; Grafstein & Laureno 1973; Wiesel, Hubel & Lam 1974).

In situations where this approach is viable, adequate control experiments are of absolute necessity. As in the demonstration of sparse projections, large injections of high concentrations and long survival times are generally required for transneuronal labelling. These

features also present a number of opportunities for false positive data. Accompanied with the delivery of relatively large quantities of tracer is the potentially large volume of tissue to which that tracer may be made available for uptake and transport. Similarly the higher the concentration of the tracer, the greater the likelihood of increasing the size of the injection site due to the proportionally greater diffusion forces present.

These factors therefore need consideration in limiting the administration of tracer solely to the projections being mapped. The more extensive the site of delivery, the greater the chances of uptake by neurons outside the scope of the study and also by the circulation and CSF. "Non-specific" uptake from these latter components could well lead to erroneous identification of structures within the vascular and ventricular walls.

A feature of OAAT which has proved of great advantage to neuroanatomists is its apparent relative immunity from the "fibre of passage" problem. In view of the paucity of ribosomes in axonal processes, it would not be surprising to find that intact or damaged axons do not incorporate externally applied labelled amino acid into protein and confuse the translation of ARG data (Cowan et al 1972).

In contrast to degenerating neural elements, the detection of which is dependent upon atrophic "changes" in the structures, light and electron microscopical identif-

ication of autoradiographically-labelled efferents allows observation of the normal morphology of the features. Also, the efferents remain labelled for a relatively long period after tracer injection so survival time does not require the empirical assessment upon which experiments detecting degeneration are so dependent.

Horseradish peroxidase

The use of a tracer technique in the form of autoradiographic detection of axonally-transported labelled material clearly represented a methodological revolution for neuroanatomy. Its conception created an intellectual and practical spur for "tracer technology".

Despite its innovativeness however, two practical drawbacks accompany its utilisation. Primarily, its usefulness is generally confined only to identification of efferents from its site of application. Even this has been questioned with the consequence that data may be confused by the presence of afferent labelling. Secondly, the method of tracer detection involves various "manipulative" stages and a relatively long exposure period.

The development of a method based on the enzyme, horseradish peroxidase (HRP) avoided these practical difficulties. Additionally, it allows direct observation of the tracer <u>in situ</u> rather than of an "event" resulting from its presence, as in the ARG approach.

The original use of HRP in tract tracing was to supplement OAAT and degeneration data by identifying the afferents to the site of injection (reviewed by Winer 1977). The development of the technique has been chronicled elsewhere (Mesulam 1982) but, briefly, it derives from experiments on the stability of the complex created between HRP, its substrate and an index of this reaction (Straus 1964; Graham & Karnovsky 1966). The

resulting availability of a comparatively rapid procedure for histochemical detection of HRP and the subsequent observation of retrograde transport of the enzyme in spinal motor neurons (Kristensson & Olsson 1971) established the viability of HRP as a neuroanatomical tracer. Later improvements in sensitivity of the detection procedure and means of facilitating uptake have revealed an orthograde component of intra-axonal HRP transport and the method has consequently evolved to enable differentiation of both afferents and efferents of a particular structure.

The HRP technique therefore combines expedient features of its predecessors with advantages of its own: 1. Both, the sources of afferents to, and the projections from the region of interest can be demonstrated using a single procedure. 2. Tracer detection is a first-order process and the histochemical procedure itself provides results relatively rapidly and consistently. 3. The tracer appears to have little or no immediate toxic effects on the neurons and the normal morphology is thus sustained and observable (Bishop & King 1982).

The majority of the experimental studies reported here have utilised these advantages of the HRP method. The details of the various features will therefore be discussed in detail beginning with aspects of the detection process.

Histochemical detection of HRP

The detection of transported HRP *in situ* is accomplished by visualising the reaction beteen the enzyme and its substrate (peroxide). This is made possible by the availability of a variety of compounds (chromagens) which react with the enzyme-substrate complex precipitating a coloured reaction product at the site of enzyme activity.

A number of appropriate chromagens have been applied to this purpose but the basic reactions are similar for all the procedures (Mesulam 1978).

$$HRP + H_2O_2 = [HRP.H_2O_2]$$

$$[HRP.H_2O_2] + (CHR)H_2 = HRP + 2H_2O + CHR\downarrow$$

Most of the chromagens which have been used are derivatives of benzidine, and the range used reflects attempts to increase the sensitivity of the histochemical reaction. Maximum sensitivity is clearly necessary in order to optimise the demonstration of neuronal connections.

The original histochemical method used was based on diaminobenzidine tetrahydrochloride (DAB) as the chromagen (Graham & Karnovsky 1966). A number of techniques, involving DAB but modifying additional parameters of the incubation procedure such as the nature of the buffer, the temperature and the stabilisation of the reaction product, subsequently evolved (Lavail & Lavail 1974; Adams 1977; Streit & Reubi 1977; Malmgren & Olsson 1978).

Other derivatives such as benzidine dihydrochloride (DeOlmos & Heimer 1977; Mesulam 1976), o-dianisidine (DeOlmos 1977) and o-tolidine (Somogyi, Hodgson & Smith 1979) have also been introduced. In addition, non-benzidine derivatives such as p-phenylenediamine and pyrocatechol (Hanker, Yates, Metz & Rustioni 1977) have been used.

However, in comparisons of histochemical methods for HRP detection (Mesulam & Rosene 1979; Morrell, Greenberger & Pfaff 1981; Olsson, Arvidson, Hartman, Petersson & Tengvar 1983) the general consensus has been that the most sensitive procedure is that using tetramethyl benzidine (TMB), another benzidine derivative (Mesulam 1978; Hardy & Heimer 1977; Mesulam, Hegarty, Barbas, Carson, Gower, Knapp, Moss & Mufson 1980).

The introduction of this method, as well as providing a more sensitive substitute for previous chromagens, provides a safer alternative. The carcinogenic properties of benzidine and several of its derivatives present a hazard in their use. The preparation of tetramethyl benzidine and its apparent lack of carcinogenicity (Holland, Saunders, Rose & Walpole 1974; Garner, Walpole & Rose 1975) thus provides a suitable substitute.

In quantitative studies, use of the TMB chromagen was observed to be superior to other methods as judged by counts of the numbers of perikarya sequestering retrogradely-transported HRP (Mesulam & Rosene 1979;

Morrell et al 1981; Olsson et al 1983). Additionally, qualitative assessment revealed better detection of orthogradely-transported HRP using TMB than the other procedures, which in some cases, notably employing the DAB method, failed to demonstrate efferent projections. The lack of sensitivity (using DAB procedures) for the quantities of HRP which were orthogradely-transported probably accounts for the initial claims that the tracer was not significantly transported in that direction and therefore confined to a role as a retrograde tracer.

The effectiveness of HRP as a neuroanatomical tracer can thus be seen to depend largely on the chromagen employed in the histochemical reaction, TMB being the most favourable. The efficacy of TMB itself, however, is subject to the influence of a number of variables at successive stages in the detection procedure. Optimum detection relies not only on the chromagen but also on the concentration of reagents in the enzymatic incubation and various perfusion-fixation parameters.

Since the introduction of TMB, the regimen of the method has undergone manipulation of a number of these features in order to obtain maximal visualisation of the tracer location whilst minimising artifactual deposits.

Prior to histochemical detection of the transported tracer in the tissue, a first requirement is vascular perfusion with a physiological saline solution. This procedure removes erythrocytes from the circulation and is

necessary because their endogenous peroxidase activity would otherwise confuse or obscure the experimental data. Previous administration of sodium heparin (or addition to the perfusate) is frequently used to facilitate this removal and reduce the possibility of clotting.

The nature and concentration of the fixative subsequently perfused through the tissue has been found in a number of cases to be an important determinant of the chromagenic sensitivity. Several reports on the effects of aldehyde fixation upon HRP detection suggest that 1% paraformaldehyde (in combination with 1% glutaraldehyde) preserves enzyme activity and its subsequent reaction product whereas greater concentrations of paraformaldehyde lessens the possibilities of detection (Jones & Leavitt 1974; Kim & Strick 1976; Malmgren & Olsson 1978).

In a comparison of fixation variables (Rosene & Mesulam 1978), it has been found that extended exposure to fixative (12 hours) abolishes HRP activity in many neuronal populations. Also, excess, unbound fixative was observed to inhibit the histochemical reaction itself. In contrast, it has also been reported that fixation periods of up to 47 hours have no detrimental effects on detection of retrogradely-transported HRP (Morrell et al 1981). However, this work made no mention of the possible consequences of extended exposure to fixative on the detection of HRP transported somatofugally.

In general, a solution of 1% paraformaldehyde-1.5%

glutaraldehyde in 0.1M phosphate buffer at pH 7.4 or 2.5% glutaraldehyde in the same buffer is used for fixation. A postperfusion immersion in fresh fixative for two to four hours is frequently added.

As frozen sections are generally required, immersion in 20-30% sucrose solution for cryoprotection precedes sectioning on a freezing microtome. It has been suggested that extending this period to several days has no deleterious consequences for the sensitivity of the histochemical reaction and may in fact provide improved morphological detail (Mesulam 1982). After cutting, the sections are rinsed in phosphate buffer at pH 7.4. They may be stored at this stage for several days prior to the reaction because the tissue-bound enzyme activity is stable at a neutral pH value.

Correct management of the parameters of the sections' enzymatic incubation is clearly crucial to the goal of obtaining optimum detection. Variations in incubation time and reagent concentration result in significant differences in the extent to which the enzyme can be demonstrated. In addition, these deviations can result in varying degrees of inconvenient artifactual deposits.

The essential factors requiring control include:

a. Buffer identity and pH value.

Several constants of the reaction equation vary markedly with acid-base balance affecting the degree of

precipitation of chromagen. For TMB a pH value of above 5.0 depresses sensitivity whereas pH2.5 or below affects the tissue integrity. Acetate buffer at pH3.3 is usually employed (Mesulam 1982).

b. Alcohol concentration:

A number of chromagens (including TMB) are virtually insoluble in aqueous media and they are therefore dissolved in alcohols. However, in the case of TMB, the ultimate product of the histochemical reaction is sensitive to alcohol presence and so a balance between the twin requirements of solubilising the chromagen whilst avoiding depression of the overall sensitivity is required. Thus, each 15mg. of TMB is initially dissolved in 7.5ml. of ethanol (Mesulam 1978). This meets the solubility requirement and the concentration of ethanol is considerably reduced when the solution is added to the reaction medium.

c. Temperature of the reaction medium.

In a similar manner to other enzymes, HRP activity is temperature sensitive. Reaction at room temperature (19-23°C) has been found to be consistently successful.

d. Incubation period.

Duration of incubation requires a compromise between maximising HRP detection and minimising artifact in the background. Periods of reaction which are too short result in depressed sensitivity even if adjustments in the temperature, and the concentrations of substrate and

chromagen are made to compensate for the brief exposure. Extended incubation periods, on the other hand, have been demonstrated to increase the spurious artifactual deposits. A twenty-minute reaction time has proved to be a suitable balance between the two needs (Mesulam 1978).

e. Substrate concentration.

Although the presence of the substrate, H_2O_2, is clearly necessary for the detection of the enzyme HRP, the precise concentration required for optimum visualisation appears to vary amongst the numerous preparations to which the method has been applied. Again, the balancing act of detection versus artifact must be considered and the appropriate concentration assessed from trial experiments.

f. Stabilisation of the reaction product.

If the product of the reaction between enzyme, substate and chromagen is not stabilised it rapidly disappears from the sections. The precise causes are unclear but the addition of sodium nitroprusside (nitroferricyanide $Na_2Fe(CN)_5NO$) to the incubation medium prevents this transience (Straus 1964). The same compromise between insufficient and excess also applies to the concentration of this reagent in the stabilising solution. Nonspecific precipitation of reaction product can be decreased by reduction of the nitroprusside concentration but this may also depress overall sensitivity (Mesulam et al 1980). Careful adjustment towards the optimum concentration is thus equally important at this stage in

the detection process. A solution containing 100mg. sodium nitroprusside in 92.5ml. distilled H_2O and 5ml. 0.2M acetate buffer at pH3.3 (or multiples thereof) has become a standard combination (Mesulam 1978).

Prior to this incubation-stabilisation reaction the sections are exposed to a presoak in an equivalent medium but excluding the substrate. Although the necessity of this procedure does not seem to have been empirically evaluated it does appear to be intuitively sound, allowing complete infiltration of the tissue by the reagents in advance of enzymatic incubation.

Recently, variants of the stabilising medium have been introduced for different reasons. Due to the presence of a nitroso (NO) group, sodium nitroprusside includes a toxic potential and in laboratories where the HRP method is often employed the hazards are self-evident. Potassium ferricyanide, $K_3Fe(CN)_6$, has been shown to be a suitable alternative stabilising agent. Its use avoids the danger of nitroprusside whilst retaining the ability to stabilise the reaction at low concentration with low levels of background artifact (Albers, Klooster & Van der Want 1984).

Another modification has been suggested for stabilisation when the HRP method is combined with immunohistochemical procedures required to visualise specific neurotransmitter locations. This development has been necessary because the various parameters for optimum

detection of HRP in the sections are not generally compatible with those required for maintenance of maximum immunoreactivity. The method introduced for stabilising the TMB reaction product in this double-labelling technique involves a further incubation with the substrate in combination with DAB chromagen and a heavy metal solution (cobalt) for intensification (Rye, Saper & Wainer 1984). This produces a TMB reaction product which is not only stable when exposed to alcohols for long periods but also in aqueous solutions at neutral pH value. The sensitivity is also comparable to the standard TMB procedure. Due to the stability in alcohols, this method may also become routinely used to prevent loss of reaction product during the dehydration necessary for long term preservation of standard TMB-reacted sections.

It is quite evident then, that a great deal of effort has been devoted to maximising the sensitivity of the histochemical procedures for the detection of transported HRP. The methodology has proceeded through a number of developmental stages and it would seem likely that certain of the variables will be continually subject to "fine-tuning".

As concerns the actual observation of the TMB-HRP reaction product at the light microscopical level, various optical systems have been utilised to optimise its contrast with the adjacent unlabelled tissue.

A quantitative comparison of brightfield versus darkfield illumination does not seem to be available and the general impression is that the various investigators differ in their preferences. However, the darkground contrast approach does appear, at least subjectively, to offer superior detection of the smaller accumulations of reaction product granules. In consequence, this is often employed to reveal orthogradely-transported HRP. The use of polarisation microscopy is growing because it has been found to be even more efficient at revealing terminal projections when TMB has been employed as the chromagen. This approach is not suitable for detection of DAB reaction product however, as this does not rotate the plane of polarised light (Hess & Schneider 1981).

Alternatively, brightfield contrast is essential to observe labelled neurons in relation to the cytoarchitectonics of their counterstained background. In addition, and especially in the case of retrogradely-labelled perikarya, brightfield illumination makes available more of the morphological details.

The various forms of contrast thus have their individual advantages and disadvantages, and the choice between the alternatives is governed by the features of interest.

Tracer delivery and injection site

A discussion of these subjects in conjunction is relevant because there is a direct causal relationship between methods of delivery of the tracer and the extent of the resultant injection site. The conclusions from an experiment employing tracer methodology are inextricably linked to the volume of tissue exposed to the tracer compound. If the site of delivery involves an area or areas not included within the goals of the investigation, the experimental results may incorporate "contaminated" data. For this reason, much care is required to contain the tracer within the limits of the structure being studied.

A detailed and lucid account of the factors which influence the size of an HRP injection site has been provided by Mesulam (1982). However, a summary of some significant points will be relevant in the context of the following discussion.

Firstly, absolute discrimination of the volume of tissue from which uptake and associated transport of applied HRP proceeds is not straightforward. Physical and chemical processes subsequent to injection usually preclude a definitive evaluation.

Following histochemical processing, the original site of administration of the exogenous HRP is generally

observed to have an intensely-labelled central zone where morphological features are indistinguishable. On the periphery of this, and extending for varying radii, is a less reactive region where neural features are visible but nonetheless characterised by a grainy deposition of reaction product.

Observations of the extent of the injection site have revealed distinct differences following systematic variations of survival times (Hedreen & McGrath 1977; Vanegas,Hollander & Distel 1978; Fahrbach, Morrell, & Pfaff 1984). The size reaches a maximum initially and then decreases with time (possibly disappearing altogether in experiments requiring long survival periods), leaving the diffuse reaction product surrounding the dense core. Some differences exist between the studies as far as the precise timing of these events is concerned but the general pattern is consistent. The maximum spread seems to occur prior to the first 6-12 hours, followed by a rapid contraction to a radius of possibly one third or one quarter of maximum after 24 hours.

The actual volume of tissue initially exposed to the tracer is thus probably greater than that circumscribed by the area of dense reaction product. The diffusely reactive surround may represent a combination of the real extent of exposure plus locally-transported tracer.

However, some confusion exists as to the limits of

the effective, or functional, transport zone within the observable injection site. Some studies suggest that it corresponds approximately to the region of intense reactivity (Jones & Leavitt 1974; Vanegas et al 1978; Mesulam 1982). Others however have proposed that transport can occur from both the inner region and the outer diffuse site (Hedreen & McGrath 1977; Ahlsen 1981). Uptake from the outer zone is not unlikely because endocytosis of the enzyme has been demonstrated as rapidly as 15 minutes after application (Turner & Harris 1974), i.e. at a time when the expanding injection site may be attaining its maximum size (Fahrbach et al 1984). To add to the interpretive difficulties, axonal terminals, dendrites and perikarya may differ in their ability to incorporate HRP (Broadwell & Brightman 1977).

Definitive evaluation of the effective site of injection is thus disputable especially with lengthy survival times. In order to avoid overestimation of afferent or efferent connections it is important to note that, after histochemical processing, the true site of tracer administration may not correspond to the immediately demonstrable reaction product. If any overestimations are to be made, they should be directed towards the extent of the region exposed to the tracer.

The sensitivity of the histochemical processing itself is also crucial to the determination of the effective injection site. As already shown in the discussion of

tracer detection, the degree of visualisation of transported HRP varies with different features of the processing. Evaluation of the zone of uptake is equally influenced by the same requirements for optimal detection. The use of less sensitive methods would probably result in an underestimation of the spread of the tracer at the site of administration.

In addition, equivalent optical conditions are needed for microscopical observation of the reaction product at the injection site as are necessary for optimum discrimination of the reaction product of transported HRP.

Concerning the various means of application, a great deal of attention has been directed towards this issue because the actual method of delivery determines the primary distribution of the tracer. This initial spread is influenced by the concentration of the tracer and the electrostatic and hydrostatic forces on its molecules (Mesulam 1982). Additionally, in the case of pressure injections, the volume and rate of ejection will affect the immediate distribution. The greater the volume and the faster it is injected, the wider the spread of tracer in the tissue.

A variety of systems have been developed for the "injection" of tracer substances, some simple and others of a more elaborate design.

The constraints placed on the design of such a

system are:

1. Stability of the injection apparatus.

This is particularly relevant to procedures involving injection into central nervous sites. Here, even slight movement during application may result in misdirection of the tracer and also cause tissue damage thus effectively increasing the desired size of the injection site or even labelling the wrong site.

On the other hand, the system needs to incorporate manouvrability in order that it may be accurately positioned, stereotactically or otherwise, in a number of sites. These requirements can be met by mounting the injection device in a rigid micromanipulator.

2. Accurate control of injected volume.

It is clearly desirable not only to employ a system which provides a means for determining the volume of tracer administered but also allows reliable duplication of that volume.

In the simplest case, commercially-available, low-volume, calibrated microsyringes can be used to these ends. However, the outside diameter of the needle is large and, in many instances, during penetration of the area of investigation is therefore likely to cause damage of fibres. An added disadvantageous feature of their use is the tendency for the tracer to leak back up the needle and

thus expose a large volume of tissue in irrelevant areas. The use of such devices is consequently limited mainly to injection of HRP into superficial CNS sites and peripheral structures.

For deeper CNS regions, systems have been fabricated using micropipettes because of their smaller tip diameters. These may be attached to a calibrated source directly (Fish & Rhoades 1981) or indirectly (Shipley 1982) or measurement of ejected volume can be made by viewing the HRP meniscus through a dissecting microscope fitted with a measuring reticle (Saper 1983). Alternatively, volume can be determined without observing the fluid by using a stepping microdrive to depress the plunger. Relating the known movement of the plunger to the internal diameter of the micropipette allows a calculable quantity to be ejected (Meyers & Snow 1981).

The main source of problem associated with the use of these sorts of pipette-based devices is blockage. This can be overcome, nevertheless, by breaking the tip to give a wider diameter through which delivery is unobstructed.

3. Precise control of injection rate.

Some of the devices already described have the capacity to allow slow ejection of HRP into the tissue. The use of a microdrive in particular provides relatively fine control over the rate of depression of the plunger.

Other assemblies using pneumatically- or

hydraulically-generated pressure have been developed which give quantifiable control over infusion rates (e.g. McCaman, McKenna & Ono 1977; Amaral & Price 1983; Tamamaki, Watanabe & Nojyo 1984).

The issue of tip blockage is even more relevant in these systems as it would produce a transient, but significant, rise in pressure which when released may cause ejection of a particularly large volume of HRP. Rigorous preparation including filtration or centrifugation of the tracer are thus required when employing this approach.

So, in general, although these latter systems appear to be superior mechanical devices, efficient control can be accomplished using the "microdrive method". With sufficient care and patience, reproducible nanolitre volumes of HRP may be injected at a rate which avoids reflux along the channel created by the advancing microsyringe.

Ionophoresis

The deposition of small volumes of HRP into sites situated deep in the CNS is frequently accomplished by utilising the ionic charge of the HRP molecule in solution (Graybiel & Devor 1974). The passage of current through a micropipette containing HRP in a salt solution results in the delivery of the enzyme to the region surrounding the tip of the micropipette. The quantity of HRP deposited in

this manner is dependent upon the current level and the time for which it is applied: a specific current value delivers larger quantities of HRP with increasing periods of time. An advantage of this method is that very small volumes of the tracer can be delivered and consequently, injection sites can be particularly punctate.

In addition, although the localisation of a particular structure within the CNS can be fairly accurately accomplished by stereotactic means, electrophysiological localisation is more precise. Thus, the response of the region under investigation can be monitored by recording through an HRP-filled micropipette and when the response has been optimised the ionophoretic current is applied. In this way the likelihood of accurately delivering the tracer is greatly increased.

The ionophoretic administration of tracer also allows the use of smaller tip diameters than mechanical devices using micropipettes. In conjunction with a narrow shank this approach minimises any mechanical damage to the structure under investigation. Consequently, it also decreases the likelihood of disrupting axons merely passing through or close to that structure.

Evidence suggests that axoplasmic incorporation of HRP rarely occurs via intact axonal surfaces (LaVail & LaVail 1974). In contrast, HRP is readily taken up and transported by the exposed axoplasm of damaged axons (Malmgren & Olsson 1979). Thus, it is quite evident that

tracer delivery procedures which also impart tissue damage may lead to data contaminated with "fibres-of-passage" label.

This fibre-of-passage problem is clearly a critical issue to be confronted for accurate determination of neuronal connectivity. Therefore, for the purposes of tracing the connections of structures lying deep within the CNS, the delivery of HRP through fine-tipped micropipettes appears to provide the optimum means for minimising the problem. Although mechanical devices equipped with micropipettes are used, the best approach, in terms of accuracy of delivery and minimal damage, is one utilising electrophysiological localisation of the relevant structure with subsequent ionophoretic HRP delivery.

One of the major advantages of the ionophoretic approach is the facility to minimise tissue disruption by the use of a very small tip diameter. It would therefore be counterproductive to negate this advantage by using high current values for HRP delivery as they might produce tissue damage. Currents of 5μA or below are generally used for extracellular ionophoresis.

The main problem associated with this method of delivery is blockage at the tip of the micropipette. However, this can be prevented by avoiding the use of high concentration HRP solutions and applying the current in pulses rather than continuously. Alternatively, micropipettes with larger tip diameters can be employed but

this latter option can increase the chances of labelling "fibres-of-passage".

There are other means by which HRP can be applied. As a paste it can be administered to superficial sites, for example, on the end of an insect pin (e.g. Finger 1978). In this semisolid state it can also be applied to the cut end of a transected fibre bundle (e.g. Katz & Karten 1983). HRP pellets or "chips" have also been created for similar purposes by allowing the semisolid tracer to dry to a solid mass and then fragmenting it into the required shapes and sizes (e.g. Griffin, Watkins & Mayer 1979).

The advantages of this form of the tracer, as opposed to aqueous solutions of HRP, for superficial or cut-nerve applications are twofold. Firstly, the "experimental" site is exposed to the maximum available concentration of the enzyme and secondly, the surrounding surface of the brain or neighbouring structures are not bathed with tracer-laden fluid.

Maximum concentration of HRP at the injection site can also be obtained by injecting the tracer in its crystalline form (Weller & Smith 1981). Again, this may well be adequate for labelling structures near the surface of, or outside, the CNS, but expelling crystalline HRP directly into deep sites does not allow control over volume or delivery rate and is likely to disrupt the tissue in the area of injection.

A disadvantage of applying solid preparations of HRP to nervous tissue may be the presence of osmotic forces tending to dehydrate the tissue surrounding the site of application. In these circumstances, the integrity of the neurons under investigation may be questionable.

Tracer uptake by neurons

After delivery, the efficient incorporation of HRP into the physiological transport mechanisms of the cell is crucial for its eventual detection. The ultimate efficiency of the method relies on internalising as much tracer as possible into the neurons under investigation in order to optimise its histochemical detection. That is, the more the cell takes up, the more likely it is that sufficient quantities will be transported.

The delivery of HRP into the extracellular space results in uptake by the perikarya or axonal terminals present. The available evidence suggests that this incorporation into the cell is dependent upon fluid phase (bulk) endocytosis (Trojanowski, Gonatas & Gonatas 1981). Thus, it is a passive uptake process which is a consequence of the ongoing endocytotic activity at the plasma membrane. The HRP tracer is incorporated simultaneously with other extracellular constituents and no specific binding with membrane sites is involved (Mesulam 1982). The volume of tracer taken up by cells therefore appears to rely on the degree of endocytotic activity occurring.

It has been proposed that the amount of endocytotic activity taking place at the membrane surfaces of neurons is positively correlated with the level of neuronal activity (Heuser & Reese 1973). However, in many experimental situations, elevation of the level of activity in order to facilitate the incorporation of tracer is not practicable.

Instead, in experiments where it has been deemed advantageous to artificially augment the normal uptake mechanism, a "molecular" approach has been followed. Thus, the administration of poly-L-ornithine (Itaya, Williams & Engel 1978), dimethylsulphoxide (Keefer 1978) or lysolecithin (Frank, Harris & Kennedy 1988) in combination with HRP has been shown to enhance the histochemical detection of the transported tracer. This presumably results from the facilitation of uptake of HRP whereby greater quantities of the tracer are incorporated into the relevant neurons.

The precise means by which this enhanced uptake is accomplished is unclear, but the nature of the compounds and the concentrations in which they are applied are likely to disrupt the integrity of the cell membranes. The tracer would consequently have direct access to the interior of the cell.

Regarding the use of these additives, a cautionary note deserves mention. Firstly, if their facilitation of tracer uptake is indeed dependent upon disruption of the

cell surface, the normal functioning of the cell might be expected to be compromised. As such, the results derived from such a preparation could be merely artefactual products of the method.

Secondly, the disruption of membranes within the region of administration is unlikely to be confined to those of perikarya or terminals. Axolemmal surfaces may be equally prone to damage. As previously noted, evidence suggests that endocytotic uptake of HRP does not occur at the surface of intact axons (LaVail & LaVail 1974). Therefore, in an experiment in which HRP is used without additives (and assuming no mechanical damage due to the injection procedure), labelling of fibres-of-passage by incorporation into their axoplasm can generally be discounted. If additives were included however, this assumption might be invalid.

Finally, the alternative effect of these additives may be solely to augment the normal endocytotic process. If this were the case however, neurons on the periphery of the delivery site, which would otherwise incorporate insufficient tracer for subsequent detection, may be involved. In this case the actual injection site would be extended.

The addition of these additives to the tracer solution may therefore provide questionable advantages for CNS applications. The most rigorous approach to the study of central nervous connections would seem to involve the

use of HRP alone.

Applications in the peripheral nervous system

The use of the additives for tracing connections of peripherally- coursing nerves and their branches, however, need not necessarily be restricted by the same limitations. Nevertheless, delivery of HRP plus additive into structures innervated by these nerves results in little detectable label in the central projections of the primary sensory afferents.

The nerve bundles are frequently amenable to surgical exposure. As such, transection of the bundle and application of tracer to the cut end is possible.

For the purpose of tracing motor neuron sources, the application of HRP alone to the cut end and its subsequent retrograde transport to soma is efficient. In the case of primary sensory afferents however, the experimental situation is not as straightforward. HRP administered to the transected nerve at sites distal to the ganglion is transported in the retrograde direction adequately for detection of the primary afferent perikarya. Further, anterograde, transport in the proximal segment of the neuron, however, results in inconsistent diffuse labelling of the central terminals. This seems to be due to sequestration of the tracer by the perikarya. In these cases, a second experimental intrusion, at the ganglion, is often required in order to delineate more

clearly the central relationships (e.g. Finger 1978).

The application of HRP along with one or more of the aforementioned "additives" to the cut nerve can circumvent this requirement. This extra efficiency presumably results from the internalisation of greater quantities of HRP. The presence of more of the tracer in the relevant neurons would improve the likelihood of detectable transport in the centrally-directed segments of the afferents.

Thus, a "one-step" approach to transganglionic tract-tracing with HRP is possible and is particularly suited to situations where the nerve is readily dissected from the surrounding tissue.

A further significant advance for HRP methodology, using the normal physiological processes of the cell, has resulted from the conjugation of the tracer with various plant lectins.

A number of these lectins occur and their characteristic of primary importance is the ability to bind specifically to particular saccharide groups on the cell surface (Sharon 1977). Further, once bound they have been shown to be incorporated into the granular endoplasmic reticulum of the cell. Thus, the mechanism of this internalisation has been attributed to "adsorptive endocytosis" i.e. receptor-mediated endocytosis (Gonatas, Kim, Stieber & Avrameas 1977).

The recognition of:

a. the incorporation of extracellularly-applied lectins into cellular organelles,

b. the development of a highly sensitive detection procedure for HRP, and

c. the availability of a procedure for conjugating HRP to lectins (in particular wheatgerm agglutinin - WGA), has led to the application of the WGA-HRP conjugate to tract-tracing questions.

Investigations comparing the histochemical detection of HRP to that of WGA-HRP have shown the conjugate to be much more superior. Indications are that WGA-HRP is forty times more efficient than the unconjugated enzyme for tracing retrograde connections (Gonatas, Harper, Mizutani & Gonatas 1979). For anterograde tract-tracing purposes, the conjugate has been shown to be between thirty and fifty times more efficient (Trojanowski et al 1981).

The precise mechanisms resulting in this superiority of the conjugate are unclear but are presumably due to the internalisation of greater quantities of the conjugate. However, whether this difference results from a more rapid incorporation into the cell or, among others, a slower diffusion rate away from the injection site, remains undetermined.

The lectin-HRP conjugate has also been shown to have an enhanced efficiency when utilised for tracing

transganglionic projections. Injections of 1% WGA-HRP into muscles supplied by branches of the sciatic nerve consistently resulted in dense labelling of their transganglionic dorsal horn projections. In contrast, these connections were inconsistently labelled following delivery of 40% HRP alone or with the addition of 2% dimethylsulphoxide (DMSO). Even the application of the HRP-DMSO combination to the transected sciatic nerve failed to match the intensity of label following WGA-HRP injections (Brushart & Mesulam 1980).

The advantages provided by the use of the conjugate are evident. Firstly, it is taken up and transported efficiently by intact neurons. This avoids the necessity of surgical intrusion into the structure of interest. Secondly, its extra sensitivity allows the use of lower concentrations of the tracer. This is not only important from the economic point of view but results in lower diffusion forces away from the site of delivery. Thus, the region of tracer uptake can be minimised. Finally, the smaller injection sites available can help to avoid the unwanted labelling of projections from adjacent structures.

Summary

The purpose here has been to demonstrate how the HRP methods used in the subsequent experiments have evolved and to highlight the advantages of using these

methods in tract-tracing experiments.

The development of methods allowing demonstration of specific neuronal pathways differentially from adjacent fibre bundles has enabled fairly definitive conclusions to be made regarding connectivity.

The use of a tracer substance per se has marked advantages over the methods based on myelin or axoplasmic degeneration. By utilising the physiological transport mechanisms of the neuron itself, the postoperative survival time required following tracer delivery is generally much shorter than that needed using the degeneration method in order to demonstrate neuronal connections.

Tracer delivery into CNS sites can be accomplished in such a way as to minimise the possibility of tissue damage and therefore avoid labelling of fibres of passage. The very nature of the lesioning procedures required to demonstrate degeneration actually optimises the likelihood of tracing fibres-of-passage.

The application of HRP in particular provides a number of distinct advantages, not the least of which is its consistent axonal transport in both the orthograde and retrograde directions. Thus it enables the detection of both the efferent and afferent connections of the structures within the region of delivery. Presently, this is not consistently available using other tracer compounds or degeneration methods.

The detection of transported HRP is a more reliable and straight-forward procedure than required for other methods and, with the optimum parameters, is very sensitive.

The combination of these features of the HRP method with recent improvements, which provide for more efficient neuronal uptake of the enzyme, allows the consistent demonstration of transganglionic transport. Thus, the central projections of neurons innervating peripheral structures can be readily investigated.

In view of all these characteristics, the HRP methods can be seen to supply an experimental tool which may be applied to virtually all questions relating to neuronal connectivity.

CHAPTER 2

PROCEDURE FOR PERFUSION AND HISTOCHEMICAL DETECTION OF HRP

Each independent chapter dealing with the connections at various levels of the gustatory lemniscus will detail the different surgical procedures required for the accurate delivery of the tracer in each case.

The detection of transported HRP or WGA-HRP in each case, however, was carried out using essentially the same techniques for the various stages of perfusion, fixation, sectioning and histochemical reaction.

In order to avoid excessive repetition of the parameters of these procedures, they will be dealt with in detail here. In those instances where a variation of the basic plan was introduced, the differences will be highlighted in the relevant passages.

Following tracer delivery and the subsequent period of post-operative survival required for its axonal transport, the animals were given a terminal dose of anaesthetic. This was accomplished by placing them in a 1:2000 solution of ethyl m-amino benzoate (methane sulphonic acid salt - Sigma) in water. As soon as the opercular respiratory movements ceased, intracardial vascular perfusion was performed to expel erythrocytes from the circulation and then for the fixation of the tissue. To accomplish

this an initial incision along the ventral midline was made and the two sides of the body wall were tied back. This procedure was aided by the prior construction of a suitable "fish block" fitted with metal tacks (Fig.2.1). Cotton was threaded through punctures in the muscular body wall and looped around the tacks. Increasing the tension resulted in the two sides of the body wall being eased apart, thus exposing the viscera. The viscera were then gently manipulated aside to allow clear access to the chambers of the heart.

After carefully removing the pericardial membrane, a 5cm. length of cotton was passed under the ventral aorta whilst viewing through an Olympus Stereomicroscope. A similar length of cotton was then passed under the junction between the bulbus arteriosus and the ventricle. This was then tied tightly so as to isolate the two chambers from each other and prevent venous blood from returning to the arterial system.

A small incision was made in the wall of the bulbus arteriosus and a cannula (Portex; outside diameter 0.75mm) was carefully inserted into the bulbus and thence several millimetres into the aorta. The cotton at this point was then tied tightly thus restraining the cannula in position (Fig.2.2).

After puncturing the wall of the ventricle, so as to allow the returning venous blood to escape, an initial perfusate of Young's Freshwater Teleost Saline (YFWTS -

Figure 2.1

This diagram shows the construction of the fish block for perfusion. The wooden block was carved to accommodate the shape of the animal and holes were drilled through it for fluids to drain away.
On occasions when the block was used for initial surgery (Chapter 4), anaesthetic solution could be delivered via a tube inserted in the mouth of the animal.

Figure 2.1

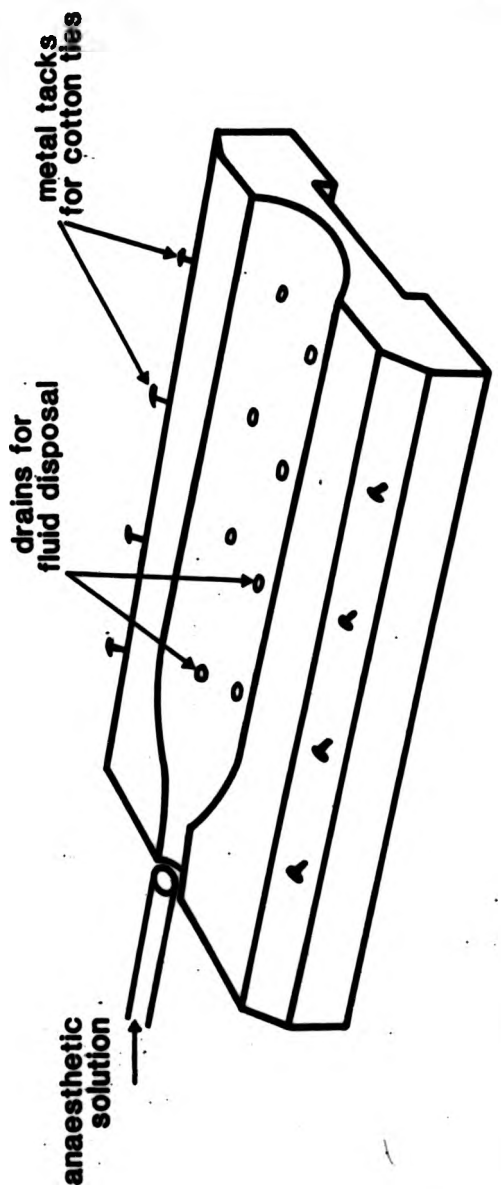
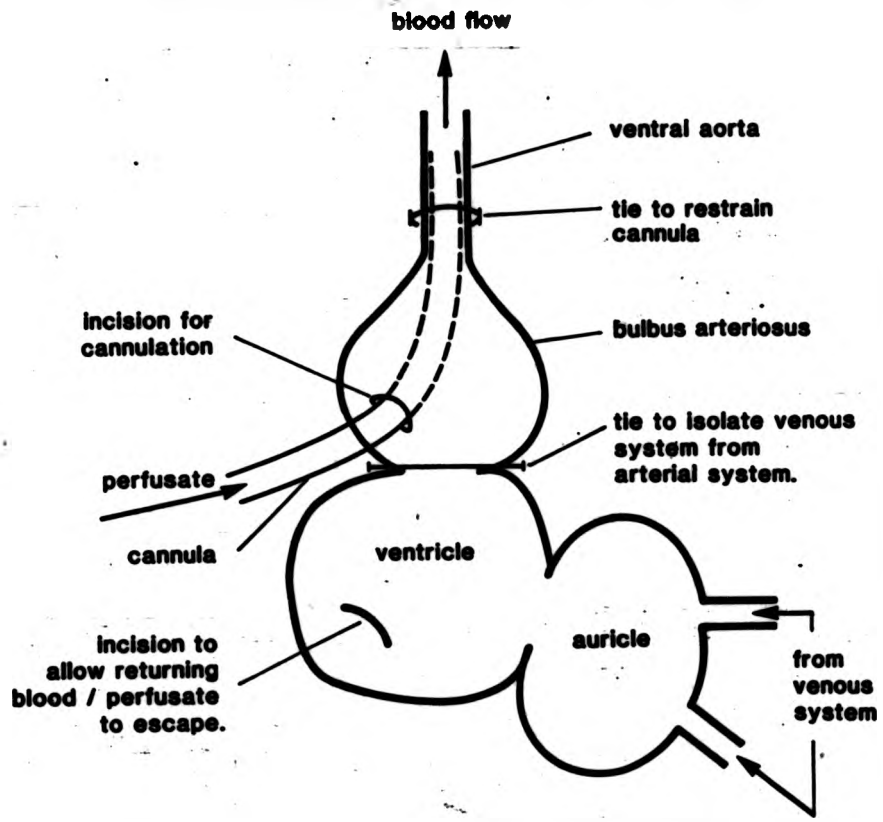

metal tacks for cotton ties
drains for fluid disposal
anaesthetic solution

Figure 2.2

This diagram illustrates the surgical procedure for cannulation and subsequent perfusion.

Figure 2.2


blood flow
ventral aorta
tie to restrain cannula
incision for cannulation
bulbus arteriosus
tie to isolate venous system from arterial system.
perfusate
cannula
ventricle
auricle
incision to allow returning blood / perfusate to escape.
from venous system

Appendix 1) at 4 °C was introduced from a 25ml. syringe (Rocket) attached to the cannula. This saline contained a 1% concentration of the anticoagulant sodium heparin (Evans).

Perfusion with this solution was continued until little or no blood was observed in the fluid escaping from the ventricle. This was generally complete after perfusion with 45-50 ml. of the heparinised saline. A useful guide to the condition of the perfusion was provided by the changing colour of the gill lamellae. Initially, before perfusion, they were a blood-filled red. This gradually changed to creamy-white following removal of the blood.

Tissues were then fixed by perfusion of the animal with 2% glutaraldehyde in phosphate buffer at pH 7.4 and 4 °C (Appendix 2). They were considered to be adequately fixed by this solution when the cardiac contractions had ceased and the colour of the gill lamellae had changed from creamy-white to yellow.

The brain and a 3cm. length of the spinal cord were carefully dissected free. Any residual tissue adhering to the surface of the brain was gently removed with damp tissue paper. The spinal cord was then transected at a distance of 3-4 mm. caudal to the medulla oblongata and retained in teleost saline.

The brain was then embedded in order to facilitate

sectioning on a freezing microtome. The embedding medium would also provide the cut sections with external support during the subsequent processing and minimise the chances of small portions of sections becoming detached. The segment of retained spinal cord was also embedded adjacent to the brain in such a manner as to identify the side of each section which was ipsilateral to the site of tracer injection.

A small petri dish was placed on a cold plate and filled to half of its depth with 20% gelatin (BDH) in phosphate buffer at pH 7.4 and 35 °C (Appendix 3). The length of retained spinal cord was held in a pair of clamps and suspended vertically in the solution on those occasions when the brain was to be subsequently sectioned in the horizontal plane (Fig.2.3A).

As the gelatin solution was solidifying, but whilst its surface remained sticky, the brain was positioned on it. It was oriented so that the suspended spinal cord segment was adjacent to the side of the brain ipsilateral to the peripheral tracer injection. The petri dish was then filled with the gelatin solution to completely envelop the brain.

Following a sufficient period (approx. 10 min.) to allow the gelatin block surrounding the brain to solidify, the segment of spinal cord was cut at the surface of the block and the petri dish was transferred to a refrigerator at 4 °C for a further 15 minutes.

Figure 2.3

These diagrams indicate the location of the embedded spinal cord segment for orientation of the tissue after sectioning.

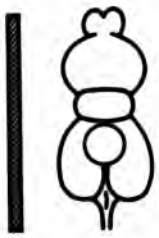
Figure 2.3

A. ROSTRAL

CAUDAL

This view from above the embedded brain indicates the positioning of the spinal cord segment (hatched) prior to subsequent horizontal sectioning.

B. ROSTRAL



CAUDAL

This view from above the embedded brain indicates the positioning of the spinal cord segment (hatched) prior to subsequent transverse sectioning.

C. ROSTRAL

CAUDAL

This view from above the embedded brain indicates the positioning of the spinal cord segment (hatched) prior to subsequent sagittal sectioning.

In instances where sectioning was to be undertaken in the transverse or sagittal planes the positioning of the spinal cord was necessarily different.

In the former case, the segment of cord was placed adjacent to the brain ipsilateral to the site of tracer delivery and along the same axis (Fig.2.3B). In the case of sagittal sectioning, the cord was positioned just rostral and horizontally perpendicular to the brain. Also, it extended on the ipsilateral side only as far as the midline (Fig.2.3C)>.

After the period of refrigeration, the solidified gelatin containing the brain was gently prised out of the petri dish and pared to form a rectangular block of suitable dimensions for sectioning. This block was then immersed in fresh fixative (25 ml.) in a screw-top bottle which was attached to a rotating plate (Taab Rotator, 4 rpm) and replaced in the refrigerator for between 2 and 4 hours.

This particular procedure was found necessary because in several earlier experiments "un-fixed" gelatin had proved fragile during section handling and mounting subsequent to histochemical reaction. The extra post-perfusion treatment with fixative was therefore carried out on the embedded brain in order to "stabilise" the gelatin support for the sections.

Following this treatment, the block was placed in

30% phosphate-buffered sucrose solution at pH 7.4 for cryoprotective purposes. It was returned to the rotating plate in the refrigerator and maintained thus until the block had submerged. For pragmatic reasons, this period generally extended overnight but even lengthier times (e.g. 3 days) had no deleterious effects on the sensitivity of the subsequent histochemical reaction.

Prior to sectioning of the gelatin-embedded brain the block was positioned on the brass stage of the sliding microtome according to the required cutting plane. Ground solid CO_2 was then gradually placed around it to cool it to a suitable temperature for sectioning. The optimum temperature for sectioning was not definitive. If the block was too cold, sections tended to fracture, and if it was not cold enough the sections varied in thickness. The skill to consistently produce undamaged sections of constant thickness was borne out of experience.

Sections of the brain were cut at 40 or 60µm. into phosphate buffer at pH 7.4 and 4 °C. The sections could be stored at this stage for up to 7 days without affecting the intensity of the HRP reaction product.

Concerning the storage and handling of the sections, a significant advance was made by the construction, in methacrylate, of a plate ("section plate") consisting of a fixed array of forty eight cylindrical

"baths" with polyester mesh bases to contain the sections (Fig.2.4A).

The advantage of this construction was that each tissue section need not be handled individually at each stage subsequent to sectioning. The array could simply be transferred from one tray of reagent to another or from one equivalently-spaced matrix of reagent-containing 10 ml. beakers ("beaker plate") to another (Fig.2.4B).

Additional advantages of this construction were that: 1) the duration of each stage of the procedure was consistent for every section, 2) large numbers of sections could be rapidly processed, 3) the serial order of the sections was maintained, and 4) in trial experiments, different concentrations and volumes of reagents could be applied to individual baths.

Summarising the stages of preparation prior to the histochemical detection of tissue-bound HRP or WGA-HRP, the overall procedure was:

1. Sacrifice by anaesthetic overdose.
2. Ventral midline incision and exposure of the heart.
3. Isolation of venous return from arterial system.
4. Cannulation.
5. Perfusion with heparinised teleost saline.
6. Perfusion with buffered-glutaraldehyde fixative.
7. Brain and spinal cord removal.
8. Gelatin embedding and orientation of cord segment.

Figure 2.4

This photograph shows the apparatus used for processing sections for HRP.

Figure 2.4

A.
Section Plate
This consists of an array of 48 baths into which the sections were sequentially placed.

B.
Beaker plate
This consists of an array of 48 beakers matching the spacing of the section plate baths.

C.
This darkened box accommodated the section and beaker plates. A similarly darkened lid provided a light-proof environment for the subsequent reaction. Matching holes in the components of the apparatus allowed their rigid attachment to a horizontal rotator.

9. Post-embedding rinse in fixative.

10. Sucrose solution rinse.

11. Sectioning into phosphate buffer at pH 7.4

Prior to histochemical processing, the tissue sections stored in the section plate were initially given four separate washes in distilled water at room temperature, with a gentle manual agitation of the section plate. Each wash lasted about 15 seconds. This was necessary to remove any glutaraldehyde remaining in the tissue as HRP shows a vulnerability to unbound aldehydes (Mesulam, Hegarty, Barbas, Carson, Gower, Knapp, Moss and Mufson 1988).

The subsequent stages of the procedure followed the protocol of Mesulam and Mufson (1988):

1. A pre-reaction soak.
2. Enzymatic incubation.
3. A wash in acetate buffer to terminate the reaction by removing unreacted reagents.

The first of these, the pre-reaction soak, involved the preparation of two separate solutions, A and B. Solution A consisted of 277.5 ml. distilled water, 300 mg. sodium nitroprusside and 15 ml. of an acetate buffer at pH 3.3. The buffer contained a 1:5 ratio of 1.0M sodium acetate to distilled water and then sufficient 1.0M HCl to bring the pH precisely to the required value. Solution B contained 15 mg. tetramethyl benzidine (TMB - Sigma)

dissolved in 7.5 ml. absolute ethanol.

As previously noted (Chapter 1), the presence of sodium nitroprusside is required to stabilise the eventual reaction product of the HRP with the chromagen, TMB.

Solution A was then added to solution B at room temperature, in a proportion of 4:0.188, to the beakers of the "beaker plate" just prior to the insertion of the "section plate". It was important to avoid adding the solutions too early in order to prevent premature oxidation of the chromagen, TMB. Equally essential for the same reason, all containers had been thoroughly cleaned by soaking in a decontaminant (Decon 90) and boiled in distilled water.

The "beaker-plate" and the inserted "section-plate" were then placed in a purpose-built box and attached to a horizontal rotator in a fume cupboard (Fig.2.4C). This pre-reaction soak was carried out in darkness to retard the spontaneous oxidation of the TMB (the exterior of the box had been painted black) and allowed to proceed for 20 minutes.

The concentration of the substrate, hydrogen peroxide, required for the next phase, the enzymatic reaction, was dependent upon consideration of both the visualisation of the transported HRP and the background artefactual density. The optimum was clearly the maximum

true label with minimum artefact.

Following several of the initial experiments, a solution of 0.85 ml. of 6% H_2O_2 w/v in 120 ml. distilled water at room temperature was found to fulfil this requirement.

Prior to the addition of this substrate solution to the baths, the "section-plate" was temporarily removed. This was essential to avoid any direct contact of the tissue sections with the substrate solution. Also, it ensured equal times of exposure of the sections during the incubation period. Immediately 0.5 ml. of the H_2O_2 solution had been added to each bath, the "section-plate" was replaced.

The incubation proceeded for 20 minutes or a shorter period if the tissue sections appeared to become excessively dark due to artefactual deposits.

On completion of the enzymatic reaction, the section-plate was removed from the baths and transferred to a tray containing chilled (4°C) acetate buffer.

This buffer was as described above but further diluted to 1:19 : buffer:distilled water. After another three washes in this buffer, the section-plate was placed in a buffer-filled tray on a cold plate and maintained at 4°C.

The sections were mounted on to slides coated with

a gelatin subbing agent (Appendix 4). Alternate sections were counterstained with 0.05% cresyl fast violet. It was essential to mount the sections fairly rapidly because exposure to the acetate buffer for periods greater than 4 hours has been reported to depress the intensity of the HRP reaction product (Mesulam et al 1980).

Once mounted, the slides were placed in a fume cupboard and air-dried for at least 2 hours, although lengthier drying times were frequently used.

The dehydration of the sections was a potential cause for loss of the reaction product but a necessary phase for preservation of long term stability. Therefore, the sections were immersed only briefly (10-15 sec.) during each stage of alcoholic dehydration. The sequence was 1) 100% ethanol, 2) 100% ethanol 3) 100% ethanol dried on silica gel. After two similar periods of immersion in CNP30 (safety solvent - BDH) to "clear" the sections, they were coverslipped using DPX (BDH) mountant.

The sections were examined and photographed under light- or dark-field illumination through a Zeiss Standard microscope. During the periods between examinations the slides were stored in a light-free environment maintained at 4 °C.

Summarizing the stages of the procedure for histochemical detection of transported HRP:

1. Four separate washes (15 sec. each) in distilled water.
2. Prereaction soak in chromagen and stabiliser for 20 minutes.
3. Enzymatic incubation with H_2O_2 for 20 minutes (or less).
4. Four separate washes in acetate buffer at pH3.3
5. Short term storage in the acetate buffer.
6. Sections mounted within 4 hours or less.
7. Air-drying for 2 hours at least.
8. Rapid dehydration and clearing.
9. Coverslipping.
10. View.

CHAPTER 3

CENTRAL RELATIONSHIPS OF THE PALATAL ORGAN INNERVATION

INTRODUCTION

The innervation of the muscular palatal organ of the carp has been associated with the highly developed bilateral vagal lobes on the dorsolateral medulla oblongata (Herrick 1905). Also, the involvement of these hindbrain structures in the feeding reflexes of the Ictalurus species of catfish has been implicated by lesion studies (Atema 1971).

The internal structure of the vagal lobes is laminar in nature and has been classically described as consisting of three major anatomical subdivisions. These are: an outer "sensory" division, an intermediate "fibre layer" and a deep "motor" division (Herrick 1905).

These divisions have been further classified in a Golgi and electron microscope study. Thus, the outer region consists of a superficial glial layer and a deeper zone which receives the primary afferent terminal arborizations. The fibre layer includes primary afferent fibres coursing into the lobe and the ascending and descending secondary gustatory fibres. The innermost zone consists of motor neuron soma and their axons. This division continues through to the ependymal surface (Ito

1971).

More recently, the structure of the vagal lobe has been given more detailed analysis in Nissl stained tissue. In the goldfish (Carassius auratus) and Crucian carp (Carassius carassius), schemes have been forthcoming whereby the laminae have been classified from superficial through to the deepest levels (Finger 1981; Morita, Ito and Masai 1988). The laminar structure of the vagal lobe in Cyprinus carpio appears to be very similar to that described for the Crucian carp (Morita, Murakami and Ito 1983). The laminar classification defined for the Crucian carp will therefore also be adopted here for the Common carp. The vagal lobe has been subdivided by Morita et al (1988; 1983) into 16 layers, numbered from superficial to deep. Figure 3.1 summarizes their laminar terminology and the cytological description of the individual laminae.

The major variations in vagal lobe structure observed amongst the cyprinoid species appear to be a consequence of differences, between species, in the relative sizes and trajectories of the primary sensory inputs to the lobe.

In goldfish, the main sensory input courses initially into the deep layers of the sensory zone and then turns outwards to project radially into the more superficial layers. A small, superficial root, known as the capsular root, courses into the lobe across the

Figure 3.1

Vagal lobe laminar classification of Morita and co-workers

	Lamina	Description
	I	Pial surface
	II	Primary sensory afferents (Capsular root)
	III	Cellular
	IV	Neuropil
	V	Cellular
Sensory zone	VI	Neuropil
	VII	Cellular
	VIII	Neuropil
	IX	Cellular
	X	Neuropil
	XI	Cellular
	XII	Primary sensory afferents (Deep root)
Fibre layer		
	XIII	Secondary gustatory tract
	XIV	Motor neurons
Motor zone	XV	Motor fibres
	XVI	Ependymal surface

surface (Herrick 1905, Finger 1981). In the lumpsucker, Cyclopterus, the main sensory afferent root penetrates the vagal lobe at the capsular level and arborizes in the superficial layers of the lobe (Herrick 1905).

In the Crucian carp, the main sensory root enters at the deepest level of the sensory division and terminates at similar levels as observed in the goldfish. The small, capsular root diverges from the main root ventrally of the vagal lobe and spreads tangentially over the whole surface of the lobe. The constituent fibres turn inwards and terminate within the same levels as the deep root (Morita et al 1988).

In contrast, in the Common carp (Cyprinus carpio), the capsular root has been described as representing the major input to the vagal lobe. The sensory afferents within this superficial layer turn radially inwards and are reported to ramify within the same region as the smaller, deep root (Herrick 1905).

Most of these reports have been based on staining of normal tissue. Although a tract tracing method has been utilised in the Crucian carp to identify the medullary connections of the vagal afferents as a whole (Morita et al 1988), the precise central projections of neurons innervating the palatal receptors alone are not known.

The purpose of this present study of Cyprinus carpio was to identify the region(s) of the vagal lobe in which the primary afferents which innervate the palatal organ receptors arborize. The use of both HRP and its conjugate with WGA was expected to allow the identification of these afferents specifically whilst leaving those inputs from extra-palatal sources unlabelled. Also, the individual efficacies of these two tracers for transganglionic transport in the primary sensory afferents of fish could be compared.

The motor division of the vagal lobe is not characterised by the same obvious laminar arrangement as the sensory layer. The motor root of the vagus nerve is reported to originate from large cells within this deep layer (Herrick 1985). This root also appears to originate from the medially-situated nucleus ambiguus (Herrick 1985), but this structure is not generally included in a classification of the vagal lobe.

The likelihood that the motor layer output provides the innervation of the intrinsic musculature of the palatal organ has been suggested by Herrick to be "extremely probable". However, following complete rhizotomy of the vagus nerve in Crucian carp, no retrograde degeneration was observed in any motor nuclei (Morita et al 1988). This method though, as described previously, has been shown to be capricious.

Experiments involving ablation of the vagal lobes of Ictalurus show disruption of the swallowing reflex (Atema 1971). Nevertheless, whether the lesions involved the complete motor layer is unclear. The disturbance of the normal feeding response could therefore have been due solely to an interruption of the primary afferent input from oropharyngeal receptors.

The present study was therefore carried out in order to identify the source of the motor neuron innervation to the palatal musculature.

Finally, the apparent involvement of the palatal organ in separating palatable from unpalatable material taken into the mouth (Sibbing 1982) suggests the presence of a rapid sensory-motor integrative mechanism. The manipulation of the material may also indicate a separate afferent-efferent relationship in the brain for each palatal surface coordinate.

Thus, this study was also designed to assess the manner in which the palatal organ of the Common carp is mapped onto the central nervous system.

MATERIALS AND METHODS

In this series of experiments, the central connections of the neurons supplying the palatal organs of 18 carp were assessed using HRP as the tracer. In addition, the same features were investigated in 25 carp employing the conjugate, WGA-HRP.

The animals were initially anaesthetised in a solution of ethyl m-amino benzoate (1:5000 in water) until the opercular movements ceased. They were then immediately removed from the anaesthetic solution, weighed and measured for length from the snout to the base of the caudal fin.

In those circumstances where the object of study was the anterior palatal region, the animals were inverted and the mouth was held open. Injections of the tracer were then made into the anterior zone of the palatal organ through the mouth.

Injections of tracer into the posterior and lateral zones were accomplished through the gill arches of inverted animals. This approach required extreme caution so as to avoid disruption of the gill filaments. Any damage to these caused excessive blood loss.

The concentration of the HRP (Boehringer Type II) injected into the carp palatal tissue was 30% in YFWTS and

was delivered via a Hamilton 5 µL. syringe fitted with a 30 gauge needle. Volumes ranged from 5 µL to 42.5 µL, those greater than 5 µL. being multiple injections into the general palatal region under investigation.

The advantage of delivering these relatively large volumes of HRP into the palatal organ was that the palatal innervation was exposed to excess of tracer. This was necessary in order to combat the difficulties associated with the transport of HRP to the central terminals of primary sensory afferents.

However, a major disadvantage was associated with this approach. The use of such relatively large volumes of tracer prevented a detailed analysis of any topographical representation due to the inevitable extensive spread of the tracer within the palatal tissue.

The development of the WGA-HRP conjugate overcame these problems and enabled the use of small volumes of discretely-placed tracer.

Injections of 1% WGA-HRP (Sigma L9008), in teleost saline or 0.9% NaCl, were made using a 5 µL. Hamilton syringe equipped with a 33 gauge needle or one of 10 µL. capacity with a 31 gauge needle. No individual injection of 1% WGA-HRP greater than 2 µL. volume was attempted as this could have led to excessive leakage of the tracer along the needle track. In those cases where the total volume delivered was greater than 2 µL., the tracer was injected into multiple sites within the region of the

palatal organ being studied.

The tracer was delivered, wherever possible, to the layers of palatal tissue immediately below the surface epithelium. The presence of the syringe needle was usually observable in the superficial tissue and local swelling of the surface at the tip of the needle indicated the site of delivery. On several occasions however, this approach was thwarted and tracer was therefore injected into deeper regions of the palatal tissue.

After all injections the syringe needle was maintained in position for several minutes prior to retraction. This also minimised the leakage of the tracer along the injection track.

Postoperatively, the animals were revived and kept in aquaria at between 17 °C and 21 °C for various periods of survival prior to sacrifice.

Tables 1 and 2 list the experimental parameters, including survival times, for each group of animals.

Table 1 (HRP injections).

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Survival (days)
CP01	78	13	30% HRP	5	3
CP02	82	14.3	"	30	3
CP03	86	14.5	"	25	2
CP04	86	14.5	"	30	2
CP05	95	15.5	"	42.5	3
CP06	88	14.3	"	30	3
CP07	93	15	"	25	3
CP08	83	14.2	"	25	3
CP09	95	15.3	"	25	1
CP10	93	14	"	15	4

Table 2 (WGA-HRP injections).

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Survival (days)
PL01	106	15	1% WGA-HRP	5	3
PL02	98	14.6	" "	5	3
PL03	108	15.5	" "	6.5	5
PL04	108	15.4	" "	7	5
PL05	94	15	" "	8.2	7
PL06	96	15.1	" "	3.5	7
PL07	75	13.8	" "	7	16 hours
PL08	89	14.3	" "	7	17 hours
PL09	81	14.1	" "	7.25	30 hours
PL10	96	15	" "	7	32 hours
PL11	85	14.1	" "	5.5	50 hours
PL12	88	14.5	" "	6.8	51 hours

Table 2 (continued)

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Survival (days)
PL13	88	13.8	1% WGA-HRP	4.5	4
PL15	128	16.5	" "	4	4
PL16	117	17.5	" "	3.5	4
PL18	126	15.5	" "	3	6
PL19	109	15.5	" "	3	6
PL20	90	14	" "	3	6
PL27	101	15.5	" "	2	4
PL29	151	17	" "	0.7	3.5
PL30	127	16	" "	2	3.5
PL31	156	17	" "	2	3.5
PL32	150	17	" "	2	3.5
PL37	93	12.3	" "	3.5	6
PL38	74	11.9	" "	4	6

The series of experiments involving delivery of 1% WGA-HRP (Table 2) included a sub-group which was designed to assess the actual spread of tracer in the palatal tissue (PL07-PL12). It was essential to clarify this issue so that definitive conclusions could be made concerning any topographical relationship between the palatal innervation and its projection to the vagal lobes.

In order to make this assessment, the palatal organs of these animals were dissected out after sacrifice. They were then subjected to the same procedure for histochemical detection of the HRP component as outlined earlier for CNS sections, but were reacted whole. They were then viewed through an Olympus stereomicroscope and the extent of the reaction product was outlined on a plan drawing of the palatal organ.

This same sub-group was also used for a brief analysis of the degree of WGA-HRP transport after various short survival periods.

As can be seen from Table 2, three short post-operative survival times were compared: one period of 16-17 hours (PL07 & PL08), one of 30-32 hours (PL09 & PL10), and another of 50-51 hours (PL11 & PL12). The six animals involved were given injections of comparable volumes.

For the purpose of assessing any topographical

relationship between the palatal organ and the vagal lobes, animals PL13, PL15, PL16, PL18, PL19, PL20, PL27, PL29, PL30, PL31 and PL32 were compared as regards the distribution of reaction product in the vagal lobes and the actual palatal zones injected with WGA-HRP. The experimental details of these animals are shown in Table 3.

Table 3

Animal number	Site of tracer delivery	Volume of tracer (μL)	survival (days)
PL13	Anterior	4.5	4
PL15	Midlateral	4	4
PL16	Midlateral	3.5	4
PL18	Anterior	3	6
PL19	Midlateral	3	6
PL28	Posterior	3	6
PL27	Posterior	2	4
PL29	Farlateral	1.7	4
PL30	Midline	2	3.5
PL31	Farlateral	2	3.5
PL32	Midline	2	3.5

From this table it can be seen that tracer was delivered to each region of the ipsilateral palatal organ on at least two occasions.

Control experiments

Three animals were used for control experiments.

Table 4

Animal number	weight (gm)	length (cm)	tracer	volume (µL)	survival (days)
C1	184	16	N/A	N/A	N/A
C2	98	14.8	1% WGA-HRP	4	2
C3	186	15.5	20% HRP	50	1

(N/A: not applicable).

Animal C1 was treated as for all other animals, without the prior injection of HRP or WGA-HRP, in order to detect any sites of endogenous peroxidase activity in the CNS. This was necessary in order to avoid confusion with genuinely-transported tracer.

Animals C2 and C3 were given tracer injections as shown in Table 3. The tracer in both cases was delivered into the bulbus arteriosus of anaesthetised animals after making a small ventral midline incision. Hamilton 5 µL. and 100 µL. syringes were used for injections into C2 and C3 respectively. Both were equipped with 33 gauge needles

which allowed easy tracer delivery into the bulbus arteriosus but avoided blood loss through the punctured wall. The body wall incision was sutured and sealed with dental cement. These injections into the circulation via the bulbus arteriosus served to control for the accidental leakage of tracer into the blood system during delivery into the palatal organ.

After sacrifice the control animals were subject to the same procedures as the experimental animals.

Control experiments were also provided by the studies using HRP and WGA-HRP to investigate the central connections of neurons supplying the barbels. In a reciprocal manner, the tracer experiments performed on the palatal organ innervation also served as controls for the investigations of the barbel innervation.

RESULTS

HRP studies

The experiments using HRP to demonstrate the central projections of the palatal organ innervation resulted in varying degrees of detectable label.

Retrogradely-labelled soma were consistently observed, bilaterally, in the motor neuron layers of the vagal lobes. The bilateral extent of the HRP reaction product however, was not necessarily indicative of bilateral innervation of the palatal muscle fibres. The delivery of large volumes of HRP was likely to expose extensive regions of the palatal organ to the tracer and may have diffused across the midline into contralateral tissue.

As will be evident from the results of the experiments utilising HRP conjugated to WGA, this indeed appears to have been the case.

In addition to the bilateral presence of labelled soma in the deep layers of the vagal lobe, the labelled cells were observed along almost the entire anterior-posterior axis of the deep layers (Fig.3.2).

On two occasions, cells in the facial motor nucleus were retrogradely-labelled, and in one animal, motor

Figure 3.2

Horizontal section showing cell bodies (MN) in the deep layers of the vagal lobe exhibiting HRP reaction product. C: caudal; R: rostral; M: medial.

Scale bar: 100µm.

Figure 3.2

neurons in the cervical region of the spinal cord were observed. As far as the facial motor nucleus was concerned, the observation of labelled cells in this region inferred that the tracer had spread from the palatal organ into the neighbouring head musculature. HRP studies of the innervation of the head musculature have found labelled cells in the facial motor nuclei (Luiten 1976).

It was likely, on the basis of this data, that the large injections of HRP had led to unlimited spread of the tracer throughout the musculature of the palatal organ (and further). The alternative explanation was that the motor innervation of the striated muscle fibres in the various regions of the palatal organ was not topographically organised in the vagal lobes.

The results of the studies using WGA-HRP will be seen to have validated the former view.

In addition to retrogradely-labelled cells, in some experiments reaction product typical of orthogradely-transported HRP was observed bilaterally in the vagal lobes. For the same reasons as previously stated, this observation of bilateral labelling did not necessarily indicate a bilateral innervation of palatal receptors.

In contrast to the motor neuron soma which were consistently labelled, the orthograde reaction product was not present in all animals. Also, when detectable, it was

generally of low density.

This feature highlighted the difficulties associated with the transganglionic transport of the tracer. Once endocytosed at the terminals, HRP is readily transported to the cell body, as evidenced by consistent labelling of motor neuron soma in the vagal lobes. However, in order to trace the central projections of primary sensory afferents from the palatal organ to the vagal lobes, it was necessary for the tracer to be subsequently transported in the orthograde direction from their ganglionic cell bodies to their terminals in the CNS.

As previously discussed (Chapter 1), HRP has been seen to be inefficient in this respect in many cases, being sequestered by the cell bodies of the bipolar sensory afferents. Certainly, this finding has been confirmed by the experiments reported here on the palatal innervation.

The orthograde reaction product, when detectable, was observed in two sensory roots at different levels in the vagal lobes. One bundle, which appeared to be the thicker of the two fibre roots, traversed the most superficial layer (Fig.3.3). The second was much more sparse and penetrated the deeper laminae (Fig.3.4). Between these two tracts could be observed the faint accumulations of label typical of orthograde transport to nerve terminals (Fig.3.4).

Figure 3.3

Horizontal section through a vagal lobe showing reaction product typical of orthogradely-transported HRP in the capsular fibre layer (CF) of the vagal lobe. Regions of dense black label (A) represent artefactual label.
Scale bar 75µm.

Figure 3.4

Horizontal section showing orthograde reaction product at various levels in a vagal lobe.
A: fibre profiles as observed in Fig.3.3 (CF).
B: terminal label (T).
C: fibre profiles of deep sensory afferents (DF).
Scale bar: 100µm.

Figure 3.3

Figure 3.4

More detailed analysis of these afferent inputs to the vagal lobe were not possible due to the paucity of data obtained from this approach.

However, the subsequent availability of the WGA-HRP conjugate, and its applicability to the "palato-vagal" system, allowed a closer scrutiny of the innervation of the palatal organ.

WGA-HRP studies.

Following injections of WGA-HRP into the palatal organ and subsequent histochemical processing for the HRP component of the conjugate, reaction product indicative of both orthogradely- and retrogradely-transported tracer was consistently observed in the ipsilateral vagal lobe.

The distinction between the different directions of tracer transport is aided by the distinct morphological nature of the accumulations of the reaction product. Thus, a fine, particulate "dust" appearance results from the presence of the tracer within terminals projected to that zone and represents somatofugally-directed movement (Fig.3.5). The density of the reaction product clearly reflects the terminal density of the projection which has taken up the conjugate. Similarly, the distribution and arrangement of the terminals within the tissue is described by the pattern of reaction product.

Somatopetally-transported tracer is quite evident from the accumulation of reaction product granules in cell bodies and their processes (Fig.3.6).

The levels of background artefact produced in these experiments were usually very low. This was possibly due to the absence of interference with the structural integrity of the circulation. The delivery of tracer into

Figure 3.5

This photomicrograph shows the appearance of reaction product which is typical of orthogradely-transported WGA-HRP. (This example is a high power photomicrograph of label in the superficial laminae of the vagal lobe - see below).

Scale bar: 50µm.

Figure 3.6

This shows the appearance of reaction product in perikarya (P) following retrograde transport of WGA-HRP. (This example is of motor neurons in the deep laminae of the vagal lobe - see below).

Scale bar: 50µm.

Figure 3.5

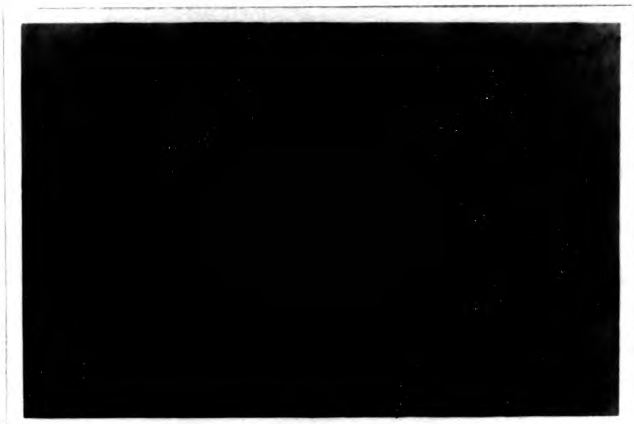
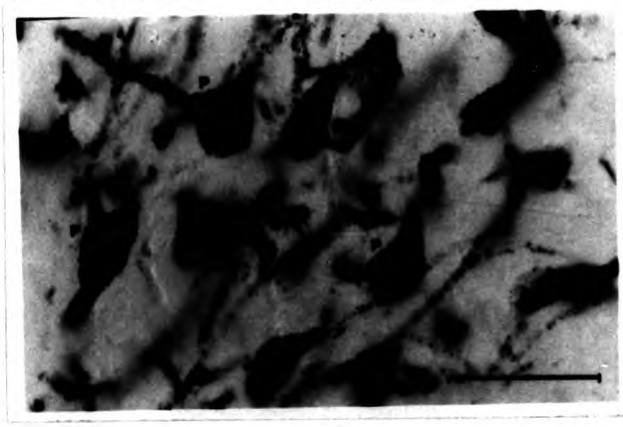


Figure 3.6



intact structures clearly results in little tissue disruption. In contrast, dissecting out the relevant nerve branches can cause much tissue damage.

The density of the reaction product following WGA-HRP injections was found to be much greater than that observed in the experiments utilising unconjugated HRP.

Reaction product was generally observed solely in the vagal lobe ipsilateral to the palatal site of administration. The only examples of reaction product in the contralateral lobe arose following injections into the midline region of the palatal organ. However, in these particular experiments it could not be positively asserted that the tracer was originally confined to one half of the palatal organ or the other.

Spread of tracer in the palatal organ

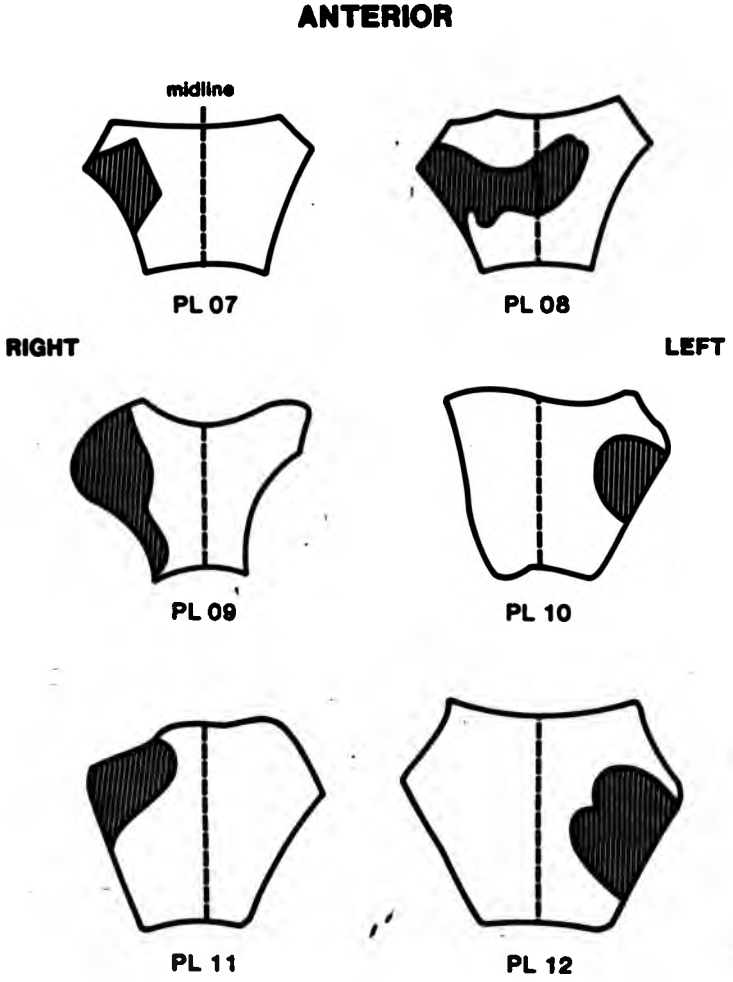
The six experiments carried out (PL07-PL12) in order to gauge the extent of the palatal tissue exposed to WGA-HRP resulted in clearly defined areas of reaction product. Injections had been carried out through the gill arches with the goal of systematically exposing all ipsilateral regions to the tracer. Figure 3.7 shows the breadth of spread of the tracer in the palatal organ following injection of volumes between 5.5µL and 7.25µL. These volumes were approximately double the amounts usually delivered in the remaining experiments.

It can be seen that in five out of these six cases,

Figure 3.7

These diagrams of the excised palatal organs of animals PL07 - PL12 indicate the limits of tracer spread following injections of between 5.5 and 7.25 µL. of WGA-HRP. The broken line in each diagram represents the approximate location of the midline of each palatal organ.

Figure 3.7


ANTERIOR
midline
PL 07
PL 08
RIGHT
LEFT
PL 09
PL 10
PL 11
PL 12
POSTERIOR

the tracer was confined to the ipsilateral half of the palatal organ. In these animals, transported WGA-HRP was found solely in the corresponding ipsilateral vagal lobe. Even in the example of PL08 where the initial injection resulted in tracer delivery to a restricted contralateral region in addition to ipsilateral tissue, no label was observed in the contralateral vagal lobe. However, the ipsilateral lobe itself was only lightly labelled in this experiment. This suggests that the parameters for optimum detection of the HRP component of the tracer were not met in the case of PL08.

Survival time

Following tracer injections, animals were allowed to survive for various periods ranging from 16 hours to 7 days.

The experiments carried out to assess injection spread (PL07-PL12) were also assessed for vagal lobe label following short survival times. Three periods were compared within this series of experiments; 16-17 hours, 30-32 hours, and 50-51 hours. The amounts injected were of comparable volumes.

Variations of length and weight were, with the exception of one animal, within 15 grams and 0.9 cm. respectively.

Survival periods of 16 to 17 hours resulted in

retrogradely-labelled cells in the ipsilateral vagal lobe. No reaction product indicative of orthogradely transported WGA-HRP was observed at these times.

The retrograde label was observed to be denser after 30 to 32 hours, the morphology of the soma being much clearer in these cases. In addition, orthogradely-labelled zones were clearly present.

The longest survival time in this series, 50 to 51 hours, resulted in the densest reaction product. Regions containing both retrogradely- and orthogradely-transported WGA-HRP were distinctly labelled. This survival time was therefore considered to be the shortest period necessary for detection of the central connections of the palatal innervation.

In practice, a period of three to six days from injection to sacrifice generally resulted in intensely-labelled efferent and afferent connections. Compared with animals which survived for 50-51 hours, the reaction product was generally denser. This occured in spite of the delivery of much smaller volumes of the tracer in most cases.

No decrease in the intensity of the reaction product was detected after the longest periods of survival (7 days). Therefore, no data on the time course of the eventual dissolution of the tracer was available.

Orthograde label

The densest accumulations of the fine particulate "dust", characteristic of orthogradely-transported WGA-HRP, were observed at two distinct levels in the ipsilateral vagal lobe. The more superficial of the two was detected in the outer sensory neuropil. As regards the second region, reaction product was distributed at a much deeper level, adjacent to, and within, the motor zone.

Fibre profiles were rarely observed with the use of WGA-HRP. This contrasted with the results following HRP delivery.

Sensory layer label

The reaction product was not confined to a single lamina of the sensory division of the vagal lobe. According to the laminar terminology adopted for the Crucian carp (Morita et al 1983), the reaction product was found predominantly across laminae V and VI with a much less dense presence in lamina VIII (Fig.3.8).

Across these laminae, the reaction product was not uniformly distributed but was observed to vary in optical density. A horizontal (Fig.3.8) and coronal section (Fig.3.9) through these layers showed a regular variation in the density.

Figure 3.8

Horizontal section of the vagal lobe ipsilateral to the injection site showing orthograde reaction product in the superficial sensory laminae. The label is found in laminae V, VI and, less densely, in lamina VIII. In addition, zones of label can be seen to be generally separated by regions with very little reaction product within them.
P: pial surface.
A: artefactual label.
Scale bar: 100μm.

Figure 3.9

Coronal section through the vagal lobe ipsilateral to the injection site showing orthograde reaction product in the superficial sensory laminae. The label can be seen to be densely accumulated in laminae V and VI. The label in lamina VIII is much less dense in this section than that shown in Figure 3.8.
P: pial surface.
A: artefactual label.
Scale bar: 100μm.

Figure 3.8

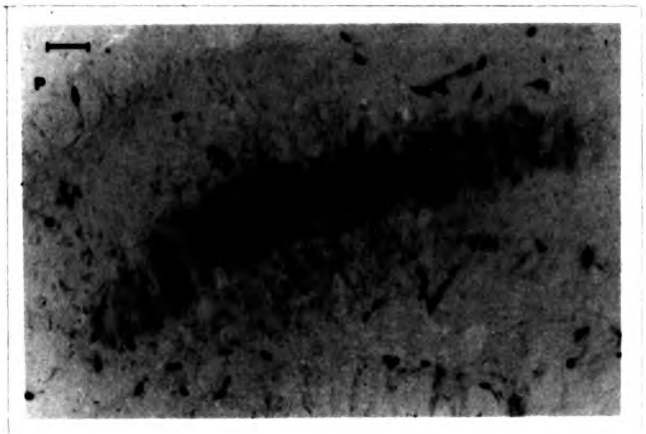


Figure 3.9

This variation consisted of bands of dense reaction product, perpendicular to the surface of the vagal lobe, alternating with unlabelled or lightly labelled intervening regions. The width of the bands of label were 15-30µm. The distance between these bands was observed to vary from approximately 50µm. to an upper limit of 200µm.

A tangential section through the labelled superficial region showed a more complete picture of the arrangement of reaction product at that level (Fig.3.10A and Fig.3.10B). The tracer can be seen to have been distributed to form a reticular arrangement. The primary sensory afferents innervating the ipsilateral half of the palatal organ therefore appear to terminate as a network across the ipsilateral vagal lobe.

The overall view of the distribution of the terminals in the superficial laminae is thus one of a generally regular, reticular or "honeycomb" arrangement oriented orthogonally to the surface. This organisation certainly appears to extend through laminae V and VI, and possibly includes lamina VIII, although the label is not clearly definable in bands at this level. The reaction product in lamina VIII may in fact represent pre-terminal axonal label.

On a number of occasions orthograde label was seen throughout the deeper levels of the sensory division in a radial orientation (Fig.3.11). Fibre morphologies themselves were not apparent presumably because of the lack of

Figure 3.18

Low (A) and higher (B) magnification photomicrographs of tangential sections through the level of the superficial orthograde reaction product. The reticular arrangement of the label can be clearly seen.

Scale bars: 100µm.

Figure 3.16A

Figure 3.16B

Figure 3.11

Horizontal section through thee vagal lobe ipsilateral to the site of injection.

A: superficial level orthograde label.

B: radial organisation of orthogradely-labelled sensory input.

C: deep layer orthograde label.

D: retrogradely-labelled cell bodies of the deepest layers.

Scale bar: 100µm.

Figure 3.11

widespread label in the axoplasmic core of the axons. The mechanism of uptake of WGA-HRP might be expected to lead to the tracer being confined to intra-axonal organelles rather than the axoplasm. However, the presence of reaction product oriented in this manner suggested that it was contained within the axons of afferents contributing the deep root.

Alternatively, this label might have been due to uptake of the tracer by secondary neurons within the vagal lobe following transneuronal transport from the primary afferent endings. This phenomenon has been observed utilising WGA-HRP in some mammalian systems (Itaya and Van Hoesen 1982). However, had this trans-synaptic transfer of tracer occurred, the cell bodies of the secondary neurons might also have been expected to exhibit label. Labelled soma in the sensory division of the vagal lobe were not observed.

Reaction product oriented in a fibre-like manner was not detected in the capsular root. The primary afferent fibres from the palatal organ therefore appear to course into the vagal lobe solely at the deep level before ramifying in the superficial laminae of the sensory division.

Motor layer label

In contrast to the orthograde reaction product observed in the superficial layers, no regular variation

in density was apparent at the deeper levels.

The label in this region was shown to be distributed in close association with the perikarya within the motor zone (Fig.3.11 and Fig.3.12). The interpretation of the presence of orthograde reaction product in this region is ambiguous. The alternative interpretations will be considered further in the Discussion.

Retrograde label

The only retrograde label observed in the ipsilateral vagal lobe was confined to the perikarya in the deepest levels of the vagal lobe and in the medulla oblongata.

Three apparently distinct groups of these cell bodies were labelled. The division of the soma into three groups was based on differences between them as regards their distribution in the vagal lobe/hindbrain neuropil, their size, and their dendritic characteristics.

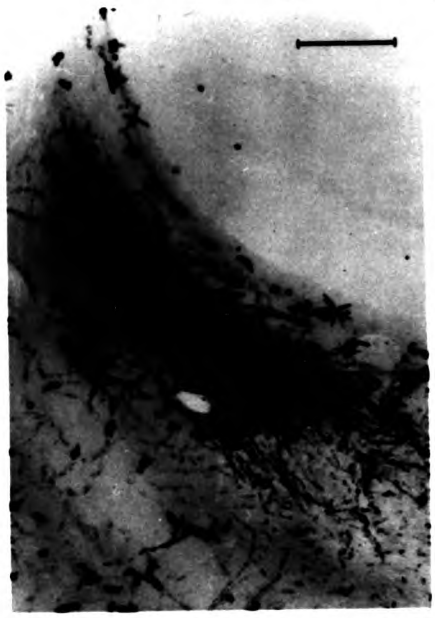
The largest group was situated in the most superficial region of the motor division i.e. lamina XIV, adjacent to the densest accumulation of deep level orthograde reaction product. The perikarya in this group were between 28µm. and 35µm. in diameter. They all appeared to possess a single primary dendrite directed radially towards, and presumably ramifying within, the

Figure 3.12

Coronal section showing labelled soma (S), dendrites (D) and axons (Ax) in the motor layer of the vagal lobe ipsilateral to the site of injection. The presence of orthograde label (O) in the more dorsal labelled region is ambiguous. It may represent afferent input to this level or densely-filled dendrites.

Scale bar: 100μm.

Figure 3.12



more superficial layers (Fig.3.13A and Fig.3.11).

A second group was observed deeper than the first group. These perikarya were located in the nucleus ambiguus (ventral motor nucleus of the vagus - Herrick 1985;1986) which, although within the vagal motor complex, is outside the generally accepted boundary of the vagal lobe. This group of labelled cells was found to occupy a less extensive range in terms of its rostro-caudal limits (Fig.3.13B). They were, in general, larger cells than those in the motor layer of the vagal lobe with diameters of between 30µm. and 50µm. Many of these cells appeared to display two large dendrites which were loosely oriented radially towards the outer surface. However, one dendrite would extend slightly rostrally whilst the other was directed in a caudal direction. This behaviour was more evident in the secondary branches.

Labelled perikarya were also observed in a medial site adjacent to the fourth ventricle (Fig.3.13C), although very few cells were labelled at this location. The diameters of these labelled perikarya varied from 20µm. to 40µm. and two main dendrites could often be observed. In some of these cells, the dendrites were outstretched along the rostro-caudal axis but oriented towards more superficial regions. Others, however, appeared to be extending caudo-medially. The site at which these dendrites ultimately ramified could not be located.

The innervation of the palatal musculature

Figure 3.13

Horizontal sections through the medulla ipsilateral to the site of injection showing the three groups of labelled soma. Top is most dorsal section, bottom is most ventral. Lateral is left, medial is right.

A: This shows the most superficial labelled group (A) which consists of the largest number of cells.

B: This shows both the superficial group (A) and a second, more medial group (B).

C: This shows both the previously described groups and a third group (C) which is both the deepest and most medial.

Further details in text.

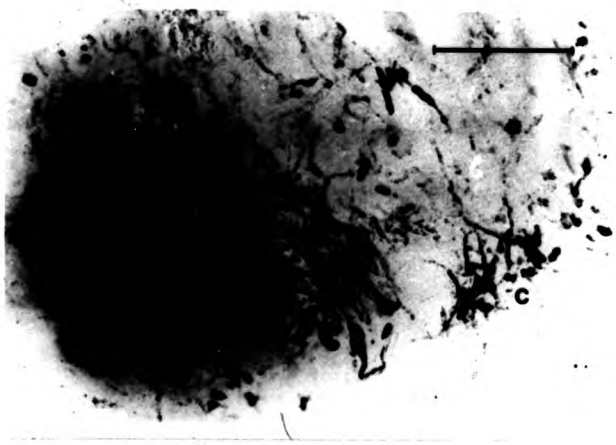
Scale bars: 200μm.

Figure 3.13

A

B

C



therefore appears to originate from three sources in the carp hindbrain. The rostro-caudal and ventro-dorsal extent of these motor neuron groups becomes increasingly limited in relation to their depth in the hindbrain neuropil.

Relating the most medial site of labelled cells (Fig.3.13C) to the nomenclature for medullary nuclei has been problematic. The main reason for this difficulty is that when these cells are labelled following injections of tracer into the palatal organ, they appear to form a separable group of neurons. They are also morphologically different from the adjacent labelled cells in the nucleus ambiguus. Thus, it seemed reasonable to regard them initially as forming a discrete nuclear population. However, after extensive observations of the tracer-labelled material and normal Nissl material, in conjunction with relevant literature, it has not been possible to ascribe a discrete identification to this cell mass.

The cells do not appear to be part of the spinal motor column as they are observed in a position anterior to the rostral limit of this column. Also, following an appraisal of the literature pertaining to a number of species, the cells do not seem to be components of the medullary reticular formation.

Following the cell group in Nissl-stained sections, it is possible to see that, at its caudalmost

extent it becomes closely associated with the more laterally-placed, nucleus ambiguus. Interestingly, at this level in the medulla of Cyprinus carpio. it has previously been suggested that the motor nucleus of the vagus consists of parvocellular and magnocellular components, the magnocellular component being at a medial position corresponding to the location of the most medially sited labelled cells in the present studies (Beccari 1943 - cited in Kuhlenbeck 1975). Two separate populations of cells have therefore been previously identified at this level. However, when labelled following tracer injections into the palatal organ, these two groups cannot be described as exhibiting a particularly clear separation into parvo- and magnocellular divisions. In fact, an attempt to describe the labelled cells merely in terms of size would probably result in a description which was, in general, the reverse of that described by Beccari.

Despite this apparent contradiction however, the convention of Beccari will be followed and the most medial labelled group of cells will be regarded as part of the vagal motor complex. In view of its close association with the nucleus ambiguus at its rostral limit, for the purposes of discussion, the most medial site will be referred to as the medial nucleus ambiguus and the more superficial group of ambiguus cells as the lateral nucleus ambiguus. It is realised, however, that this is not completely satisfactory and requires further investigation.

Topography

Following injections of WGA-HRP into the anterior region of the palatal organ (PL13, PL18), superficial and deep orthograde reaction product, and retrogradely-labelled motor neuron soma, were observed in anterior regions of the ipsilateral vagal lobe (Fig.3.14A). Also, the rostrocaudal extent of reaction product within the relevant laminae of the anterior vagal lobe corresponded at all levels, thus forming a matching "column" of label across the neuropil.

This is so although, by virtue of the vagal lobe shape, the entire rostro-caudal extents of each laminae do not match in absolute terms. It is a radial congruence of label at each level that is apparent.

Injections of the tracer into the midlateral segment of the organ (PL15, PL16 and PL19) resulted in orthograde and retrograde reaction product within the intermediate region of the vagal lobe (Fig.3.14B). Again, the labelled laminae corresponded in extent across the neuropil.

Orthograde and retrograde label were observed in the caudal zone of the vagal lobe (Fig.3.14C) following injections into the posterior quadrant of the palatal organ (PL26 and PL27). As observed following injections

Figure 3.14

Horizontal sections through the medulla showing the location of labelled sites in the ipsilateral vagal lobe following injections of WGA-HRP into:

A: anterior palatal organ.

B: midway between anterior and posterior palatal limits.

C: posterior palatal organ.

The superficial orthograde reaction product (O) is not particularly clear at this magnification. Its location is therefore indicated by arrows.

R: rostral. C: caudal.

FL: Facial lobe

Scale bars: 250µm.

Figure 3.14

already described, reaction product was confined to equivalent rostrocaudal limits within the labelled laminae.

In summary, injections of the tracer into the palatal organ resulted in columns of label across the vagal lobe neuropil, these bands being found more caudally as the tracer was delivered to more posterior palatal sites.

As regards the topographical relationship between the palatal organ and the ipsilateral vagal lobe in the medio-lateral plane, comparison of the results following injections into the midline palatal organ with those following far-lateral injections indicated different sites of reaction product.

Tracer delivered into the midline areas (PL30 and PL32) resulted in bilateral orthograde label in the most ventral region of the superficial layers of the vagal lobes (Fig.3.15A). Additionally, orthograde label in the deep layers and retrogradely-labelled motor neuron soma were observed bilaterally but in these cases the reaction product was less densely distributed than that following more laterally-directed injections. As previously noted, the observation of bilaterally-labelled sites did not necessarily infer a bilateral innervation of these palatal zones: it seems more likely to reflect the spread of tracer across the palatal midline.

Injections into the far lateral palatal organ (PL29 and PL31) produced orthograde label in the superficial laminae of the more dorsal areas of the ipsilateral vagal lobe (Fig.3.15B). Although the distinction between dorsal and ventral zones was less clear in the deeper laminae, a comparison between the regions labelled at these levels following midline and far lateral injections of tracer indicated that label in the former case was found more ventrally than in the latter case.

Briefly therefore, as concerns the medio-lateral plane of the palatal organ, reaction product was found more dorsally within the structure of the vagal lobes as the site of tracer delivery was displaced more laterally of the midline.

Control studies

All control experiments demonstrated a total lack of tracer-related label in the vagal lobes. Animal C1 exhibited no reaction product characteristic of either orthogradely- or retrogradely-transported tracer in the CNS. The only cases of endogenous peroxidase activity observed were those due to erythrocytic presence in cerebral blood vessels.

In animals C2 and C3, in which tracer had been injected into the bulbus arteriosus, no orthograde or

Figure 3.15

Coronal sections through the vagal lobe ipsilateral to the site of injection.

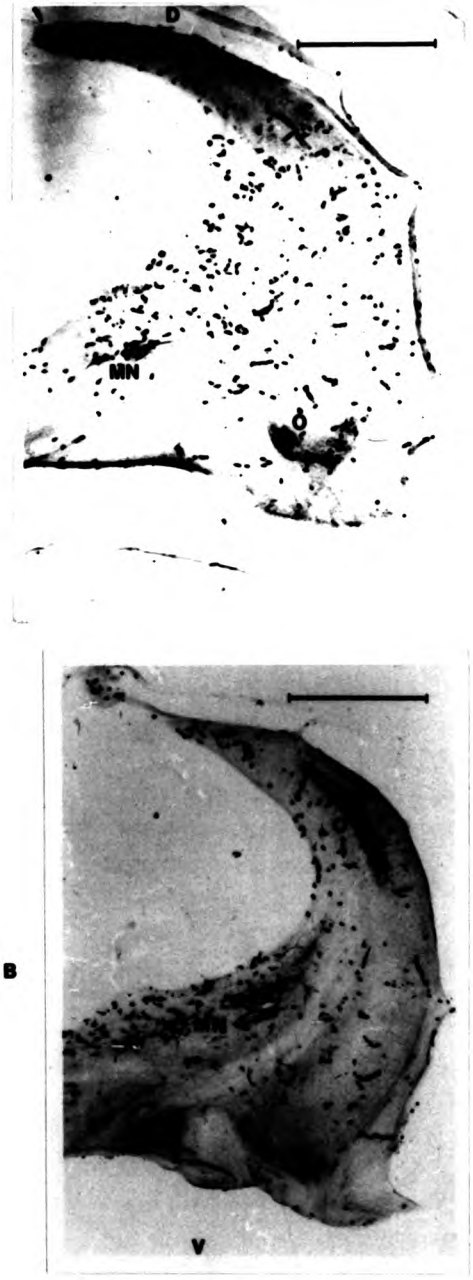
A: Tracer delivery close to the palatal midline resulted in superficial orthograde label (O) and deep retrograde label (MN) ventrally in the vagal lobe.

B: Tracer delivery into far lateral palatal sites resulted in superficial orthograde label (O) and deep retrograde label (MN) in more dorsal positions within their respective levels of the vagal lobe.

D: dorsal. V: ventral.

Scale bars: 800μm.

Figure 3.15



retrograde label was detected in the CNS. Even sites adjacent to blood vessels or the ventricular surface were devoid of tracer-related activity. These results were in agreement with other studies of lower vertebrates where an impermeability to exogenous peroxidase has been observed (Bodenheimer & Brightman 1968, Ciani, Del Grande, Franceschini, Caniato and Minelli 1983). They are, however, in contrast to the results of some investigations using HRP in cyprinoid fishes, which have shown non-specific uptake of the tracer by neurons in the preoptic region (Peyrichoux, Weidner, Reperant and Miceli 1977; Fryer and Maler 1981). Nevertheless, there were no reports of non-specific labelling in medullary regions in these studies and, as will be evident in a later chapter, injections of the tracers into the barbels did not result in reaction product within any of the regions labelled following tracer delivery into the palatal organ. Thus, all labelled zones observed following injection of tracer into the palatal organ were the result of the transport of that tracer from the site of injection.

DISCUSSION

The studies reported here have illuminated a number of cytoarchitectonic and organisational features of the cyprinoid vagal lobe.

Following injections of WGA-HRP into the palatal organ, terminal branches of primary afferent neurons innervating palatal receptors have been shown to ramify within the outer sensory laminae of the ipsilateral vagal lobe. However, the precise course of the afferent axons entering the lobe has not been definitively demonstrated. Indications from the studies utilising WGA-HRP are that these axons enter the vagal lobe solely within the deep sensory root.

However following delivery of large volumes of unconjugated HRP into the palatal organ, fully-labelled fibres were demonstrated in both the capsular and deep sensory roots. Nevertheless, in view of the injected volumes of this tracer and the simultaneous demonstration of label in central nervous regions previously shown to innervate extra-palatal structures (Luiten 1976), it could be suggested that the labelled capsular root afferents originate similarly from other extra-palatal sources.

From a gustatory standpoint, taste buds are found in other regions of the oropharyngeal cavity in fish. Thus, the possibility exists that the capsular root label observed in the experiments using large volumes of HRP may

reflect excessive spread of the tracer to extra-palatal sites and represent afferents from these sources.

These interpretations gain support from recent studies of the goldfish. In this species, primary afferent fibres from the palatal organ entered the vagal lobe entirely within the deep sensory root. Nerves innervating the gill arches however were found to course in both the deep and superficial sensory roots of the vagal lobe (Morita and Finger 1985). In the current studies, therefore, the implication is that the large injections of HRP may have allowed the spread of tracer to neuron terminals within extra-palatal oral structures.

In addition, there is a general agreement between the goldfish studies and the current studies concerning the laminae receiving palatal afferent endings. In the goldfish, palatal afferents were observed to terminate largely in lamina VI. The present experiments on the common carp have shown palatal afferent endings in laminae V and VI, and possibly also in lamina VIII, although the label at this level may be pre-terminal.

The distribution of these labelled terminals in the outer sensory laminae following WGA-HRP injections has been shown to be non-uniform. They are found in a reticular arrangement in these laminae.

A number of studies have indicated a similar organisation of chemically-identifiable systems at various levels within the sensory division of the vagal lobe.

Enkephalin-like immunoreactivity has been observed in a reticular arrangement in lamina III of the goldfish. It is also present in a less dense form in deeper sensory laminae apparently oriented perpendicular to the laminar pattern of the lobe, and in the motor layers. This enkephalin-like immunoreactivity seems to originate from a central source because sectioning the vagus nerve did not appear to diminish the reactivity (Finger 1981).

Acetylcholinesterase has also been demonstrated at various levels in the goldfish vagal lobes (Contestabile 1975; Edgar 1983; Finger 1984). In the sensory layers, it is present in a number of laminae but is densest in laminae which are known to receive the deep primary afferent input. In these laminae, the acetylcholinesterase stain exhibits a similar "meshwork" arrangement to that already described (Edgar 1983).

Substance P-like immunoreactivity is present in a reticular fashion at the level of the capsular fibre layer in goldfish. Following vagotomy, both this activity and that ascribed to acetylcholinesterase are eliminated. It seems therefore that, at least in goldfish, the deep afferent input may utilise a neurotransmitter system which is associated with acetylcholinesterase, whereas the superficial, capsular input utilises a system based on substance-P (Finger 1984).

In addition, the reticular arrangement of the deep, and superficial primary afferent terminals, and a

similarly organised central afferent projection to the vagal lobes, might suggest that this is the basic organisational principle in the superficial layers of the vagal lobe. The close association of the central and peripheral afferent terminals possibly reflects the presence of a mechanism for modulating the primary afferent input to the vagal lobe.

The reticular arrangement observed is not unlike the arrangement of terminals of lateral geniculate relay cells in the visual cortex of mammals observed following transneuronal transport of tracer injected into the eye. (Hendrickson 1985). This type of organisation of sensory systems may therefore be more widespread than previously thought. However the palatal projection in fish has not been subject to the same degree of physiological analysis as the visual projections of mammals and the functional consequences of this arrangement of terminals in the vagal lobe are therefore unclear.

In addition to the superficially-projecting afferents, reaction product characteristic of orthogradely transported WGA-HRP has been demonstrated within the motor division of the ipsilateral vagal lobe.

Whether this reflects the presence of an afferent input from the palatal organ to the motor neuron soma at this level or orthograde transport of tracer within the dendrites of the motor neurons is unclear. There are at

least two alternatives for translation of the data. Firstly, it could be interpretted that the presence of such reaction product indicates terminal arborization of primary sensory afferents in the region of the motor neuron perikarya. However, no fibre-like orientations of the label were observed coursing into the region. Nevertheless, a lack of fibre-like morphology was also characteristic of the primary afferent input to the superficial sensory layers. Labelling with unconjugated HRP, however, which resulted in visible fibre profiles in the vagal lobe following palatal injections (Fig.3.5 and Fig.3.6), also failed to reveal the presence of afferent fibres in this region.

The density of reaction product observed might suggest that any primary afferent ramification into the region would not be particularly small. This input might arise as a portion of the deep sensory root or as a separate afferent bundle coursing into the vagal lobe. However, observations of silver stained sections revealed no such input and in previous studies of normal tissue, neither axonal arborization from the deep sensory root, nor an additional fibre pathway have been reported.

A second option for interpretation of the data involves the motor neurons themselves. The close association of the reaction product to the labelled motor neuron soma, might be due to orthograde transport of WGA-HRP in the large dendrites of these neurons. Dendritic

label would be expected following retrograde transport of WGA-HRP to the motor neuron cell body. Indeed, this would represent a similar intracellular transport phenomenon to that required for the demonstration of the central projections of the primary sensory afferents from the palatal organ. The increase in density of the deep orthograde label seen with increasing survival time (PL87-PL12), could therefore be interpretted as either a gradual accumulation of orthogradely-transported tracer in the terminals of a deep afferent input to this region, or an accumulation of tracer in the dendrites which is occurring over a roughly similar time period. It could, of course, reflect the presence of both occurring simultaneously.

The observations of the orthograde reaction product within the motor layers in a coronal section of the vagal lobe (Fig.3.12) would seem to suggest that the second option i.e. that the label is dendritic in nature, is more defensible than the first. In support of this, a similar study in the goldfish which has recently been published shows no primary afferent input to the motor layers (Morita and Finger 1985).

The motor innervation of the intrinsic musculature of the palatal organ has been shown in these studies to arise from three groups of neurons in the hindbrain. Many perikarya were labelled in the true motor layer of the vagal lobe. At a deeper level in the neuropil of the

medulla, another group of cells was labelled at a location referred to as the lateral nucleus ambiguus. These cells were larger and their dendritic arborization appeared to be wider but still generally directed radially towards the more superficial layers. The third group, consisting of the fewest numbers of cells was located in a medial region of the nucleus ambiguus. The dendrites of these cells seemed to have the greatest expanse of arborization of all three groups but their polarity did not appear to be consistent.

The majority of the palatal musculature innervated by these motor neurons is organised in two planes of striated muscle fibrils loosely oriented perpendicularly to each other. The "body" of the organ consists of a matrix of fat cells embedded within the fibril network and forming an "oleostatic skeleton" (Edgar 1983).

Tactile stimulation of the palatal surface results in a protrusion of the epidermal layers within that region even in the isolated palatal organ (Cole 1944; McGlone 1977; Edgar 1983; personal observation). The function of this response in the behaving animal may be to expose a greater surface area of the organ to a particular object in the oropharyngeal cavity during feeding. Thus, the material would be made available to a larger receptive area.

It seems possible that the mechanism underlying the protrusion of the palatal surface would involve

contraction of striated muscle fibrils in the planes orthogonal to each other with the resultant distortion of the fatty tissue in that region. The muscle fibrils at superficial levels in the palatal organ have been observed to be organised othogonally to each other (see initial Introduction).

As previously noted, the protrusion can be elicited in the isolated organ. This may reflect a lack of central control of the response during feeding. Alternatively, this *in vitro* phenomenon may not be significant in relation to the response in the intact, behaving animal. If the latter hypothesis was true and in view of the contraction of muscle fibrils in two different planes apparently required to produce the protrusion of superficial tissue, the prescence of two populations of motor neurons underlying the mechanism might be expected.

Following the labelling of two such distinct groups of neurons in proximity, one within the motor division of the vagal lobe and another immediately subjacently, it might be tempting to hypothesise these two groups as candidate populations. However, there is no evidence to support this contention. Also, considering the columnar manner in which the palatal organ is represented in the vagal lobe (see below) and the apparent agonist relationship required of the two planes of musculature, there is no reason to suppose that the candidate populations would be separated within the lobe.

Additionally, in order to manipulate any material within the pharyngeal cavity, waves of contractions of the palatal musculature in conjunction with the opposing pharyngeal floor, would be likely.

To produce such an integrated sequence of muscle contractions within the palatal organ it might be expected that the motor neurons responsible would form an intimately-related "motor map" of the organ and not consist of separate neuronal assemblies.

Alternatively, if the response of the isolated palatal organ to a point stimulus is relevant to the *in vivo* condition, this implies that the immediate protrusion response is intrinsic to the organ and without extrinsic control by motor neurons in the CNS. It might be postulated therefore that the initial protrusion is a local response to a stimulus associated with the entry of some material into the pharyngeal cavity. A larger surface is thereby exposed to the gustatory qualities of the stimulus. A "map" of closely associated motor neurons may then be responsible for manipulating the material as previously described.

Striated muscle fibrils are not only present in the palatal organ at the dermal levels described. Fine fibrils are also present invading the epidermis, and attaining a close association with the base of taste buds (Edgar 1983; also see initial Introduction). Their role is unclear but their position and orientation loosely perpendicular to

the palatal surface might suggest that their function lies in the retraction of taste buds from the surface epithelium. This might be expected in the presence of a noxious stimulus.

Whether this is their true role or not, the orientation of these fine fibrils orthogonal to the plane of contraction of the deeper musculature implies an antagonist relationship between them: contraction of the fibrils traversing the epidermis would be expected to have an opposite effect to the consequences of contraction in the deeper layers.

Clearly, the data available from the present experiments cannot define the particular labelled motor neurons innervating specific muscle fibres in the palatal organ. However, it is clear that the motor neurons labelled following palatal administration of WGA-HRP are not all serving the same functional role. The varying dendritic arborizations may be an additional reflection of these differences. It would be expected that the cells of the lateral nucleus ambiguus, which appear to have a more expansive dendritic tree oriented towards the superficial regions of the vagal lobe than that of their neighbours in the vagal lobe proper, would have the capacity to accept inputs from a more extensive source. Indeed, in view of the columnar organisation of the vagal lobe, it is probable that these deeper neurons are integrating activity which originates from larger areas of the palatal

surface than the more superficial motor neurons.

The nucleus ambiguus has previously been implicated as having a role in the swallowing act in higher vertebrates (e.g. Jean 1984). The data gathered in the present experiments do not contradict a similar function for those cells within the nucleus which project to the palatal organ.

In contrast to the fine control of the palatal musculature required for the manipulation of material over its surface, the control required as a function of its involvement in swallowing is likely to be coarser. As a component of this activity, expansion and relaxation of far more extensive regions of the organ might be expected.

The labelled cells of the nucleus ambiguus were found to be, in general, larger than those in the motor layer of the vagal lobe. One of the reasons for the occurrence of large as opposed to small cells may be a greater supportive requirement in terms of the physiology of the cells. This could be due to the possession a larger dendritic tree and/or a more widespread efferent projection. Both of these might be characteristics of a motor neuron pool responsible for gross movements of the palatal organ. Inputs from more extensive palatal sources to the relevant motor neurons, and equally extensive axonal arborisations from these neurons to the musculature might be expected. Thus, a relatively small number of

large cells could be responsible for the control of the comparatively gross movements required of the palatal organ within its role as a part of the swallowing sequence. In addition, gross lesions of the vagal lobes have been reported to disrupt swallowing in Ictalurus catfish (Atema 1971).

A number of the functional hypotheses posed above are clearly speculative and provide a basis for more questions regarding the palato-vagal system than answers. This is a reflection of the paucity of studies on this particular system.

However, irrespective of precise functional roles, the present anatomical studies have demonstrated that the palatal organ is represented topographically within the vagal lobes.

The sensory and motor innervations within the anterior regions of the palatal organ are represented in the anterior vagal lobe. Increasingly more caudal zones of the organ are mapped onto increasingly caudal zones of the vagal lobe. Thus the anterior-posterior axis of the palatal organ is represented along a similar axis in the vagal lobe.

The medio-lateral axis of the palatal organ is shown to be mapped along a ventrodorsal projection of the vagal lobe. Neurons innervating medial palatal sites connect within ventral regions of the lobe whilst the

central connections of those supplying lateral regions are found in the dorsal vagal lobe. (Fig.3.16).

The gross organisation of the vagal lobe can therefore be thought of as "palatotopic". This has previously been suggested using electrophysiological procedures (McGlone 1977).

The injection of tracer into discrete fractions of the palatal organ has also revealed that the particular labelled palatal region is represented congruently throughout the layers of the ipsilateral vagal lobe. Considering the finding that the palatal organ is also represented topographically across the vagal lobes, the correspondence of labelled regions throughout the depth of the vagal lobe indicates a radial organisation of the lobe for palatal connections. Thus each palatal coordinate is represented within a "column" oriented orthogonally to the vagal lobe surface.

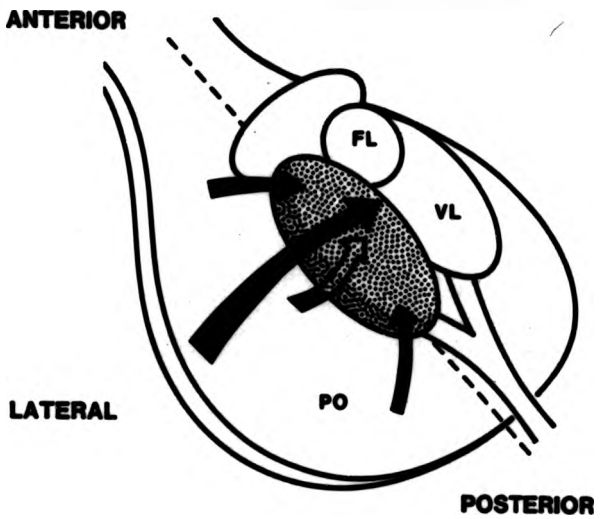
As regards the function of the system, the superimposition of the sensory and motor representation may explain the response of the organ to the presence of material on its surface. Each "sensory locus" could have connections to a correspondingly discrete motor neuron pool. The occurrence of a stimulus in a particular palatal location would therefore be expected to result in an equally localised response of the palatal musculature.

This mechanism may underlie the apparent role of the palatal organ in the sifting of material taken into

Figure 3.16

This diagram summarises the "palatotopic" relationship between the palatal organ and the ipsilateral vagal lobe. VL: vagal lobe. FL: facial lobe. PO: palatal organ. Only the medulla oblongata and rostral spinal cord is included in the diagram.

Figure 3.16



the mouth and the separation of palatable from unpalatable objects prior to swallowing (Sibbing 1982).

CHAPTER 4

CENTRAL PROJECTIONS OF THE BARBEL INNERVATION

INTRODUCTION

The Common carp (Cyprinus carpio) possesses two bilateral pairs of small barbels. The accumulations of taste buds on the flanks, and especially on these barbels, the anterior mouth and the lips result in the enlargement of the visceral afferent roots of the facial nerve in the cyprinoids and catfish (Herrick 1905).

Of the different branches of the facial nerve, three in particular have been considered to represent the main facial taste pathways. This is because these roots are believed to supply the densest populations of taste buds, on the barbels and the lips. These three roots are: 1. Ramus maxillaris 2. Ramus mandibularis 3. Ramus palatinus.

A scheme of barbel nomenclature based on the pattern of innervation would be difficult to initiate due to an apparent overlap between branches. Also, as no functional separation of the two barbels appears to exist, a system of nomenclature based on this aspect is not feasible either.

Thus the small dorsal barbel is referred to here as the nasal barbel whilst the large barbel protruding

from the upper lip is referred to as the maxillary barbel.

Associated with the strong development of the branches of the facial nerve innervating taste buds on the external body surface is an equally significant enlargement of the central nervous region receiving the afferent terminals of the system. In the carp, this enlarged region, the facial lobe, is found on the dorsal medulla oblongata. It is sometimes referred to as a "tuberculum impar", having fused with its equivalent contralateral neighbour (Ariens Kappers, Huber and Crosby 1936).

A number of anatomical studies of the facial nerve have been previously reported, not only in carp but also in other species which possess highly developed "external" gustatory systems.

The early studies on the gustatory systems of cyprinoids and siluroids by Herrick (1905) were based on the normal anatomy using predominantly the Golgi methods. Also, in a comparative study, Barnard (1936) used Nissl and silver methods to analyse the central nervous regions directly associated with the facial nerve. More recently, modern tract tracing techniques have been employed to investigate the primary projections of the facial system in a number of species.

The Fink-Heimer silver method has been used to trace central fibre connections of the facial nerve in the carp, Cyprinus carpio (Luiten 1975) and the crucian carp, Carassius carassius (Morita, Ito and Masai 1988). These

studies mapped degenerating fibres following rhizotomy of the facial nerve close to the brain.

However, by lesioning the nerve at this point, it was not possible to definitively assess the contribution of individual branches to the representation of the facial taste system in the brain.

Using both degeneration and HRP techniques, the central projections of neurons supplying the barbels have been studied in Ictalurus nebulosus (Finger 1976). This involved a two-step approach to the investigation of the relevant facial nerve branches.

Firstly, the ganglionic representation of each branch was studied by applying HRP to the cut distal end of each branch at the level of the barbel, or injecting the HRP directly into the barbel and following the retrograde transport of the tracer to the ganglionic perikarya. Secondly, the central connections were examined following discrete lesions of the ganglion and the subsequent anterograde degeneration in the proximal portion of the primary afferent neurons.

Using this approach, branches of the facial nerve innervating individual barbels were found to terminate in distinct regions of the ipsilateral facial lobe. Thus a gustatory somatotopy was observed to exist in Ictalurus.

Such a topographical organisation of the facial lobe of the carp has been reported using electrophysiol-

ogical procedures (Marui 1977). This study mapped responses in the facial lobe to chemical and mechanical stimulation of various regions of the external surface of the animal. This, however, only allowed the determination of central nervous representation at a relatively gross level. It also provided no information concerning the anatomical basis of the topography detected electrophysiologically.

In the studies reported here the question addressed was whether the somatotopy is anatomically detectable in the carp facial system following administration of tracer to the separate barbels.

To accomplish this a two-step approach was theoretically avoidable due to the availability of the highly efficient transganglionic tracer, WGA-HRP, and the sensitive chromagen, TMB. Their use also enabled the application of small tracer volumes to minimise or prevent unacceptable spread into neighbouring tissues.

In addition, the barbels being isolated structures should naturally hinder this diffusion of tracer away from the site of delivery. Thus, they also offer the opportunity for a direct comparison of the efficacy of HRP and WGA-HRP in transganglionic tracer studies of intact structures.

Since the completion of these studies a report has been published in which the central projections of

individual branches of the facial nerve of Cyprinus carpio have been traced (Kiyohara, Shiratani and Yamashita 1985).

That study applied HRP to the cut end of the distal portion of each branch and showed that they were topographically represented in the ipsilateral facial lobe. In addition, it was shown that the palatine branch of the facial nerve is purely "facial" whilst the others investigated are "trigeminofacial".

MATERIALS AND METHODS.

The investigations of the central projections of the neurons supplying the barbels involved 21 carp. Of these, 15 animals had tracer delivered to one barbel only.

In 4 other animals, separate injections of WGA-HRP and HRP were made into a different barbel of each side (see Table 5). This approach was taken in an attempt to compare directly the transganglionic efficacy of each tracer within the individual animals.

In addition, two carp had equal volumes of 1% WGA-HRP delivered into a different barbel on each side (Table 5). Assuming equivalent transganglionic transport efficiency in the case of the different neurons supplying the barbels, the purpose was to observe the central projections from each within the same animal. In this way it should be possible to observe directly any topographical organisation in the system.

The fish were initially anaesthetised in ethyl m-amino benzoate solution (1:5000 in water) until respiratory movements were no longer visible. Measurements of weight and length (snout to the base of the caudal fin) were taken. They were then loosely wrapped in damp tissue, placed on the fish box, and artificially respired by circulating water containing anaesthetic at a similar concentration to that described above.

The procedure for tracer delivery was the same for each barbel, although the delivery was easier in the case of the, larger, maxillary barbel.

A piece of cotton was loosely tied around the barbel under investigation. The tip of that barbel was grasped with a pair of forceps and the surface was firmly punctured with a broken micropipette as close to the extremity as practically possible. The needle (33 gauge) of a tracer-filled 5 µL. Hamilton syringe was then inserted into the puncture and gradually advanced into the core of the barbel. It was then slowly retracted so that the tip of the needle was close to the extremity of the barbel. The cotton loop initially positioned around the barbel was then tied tightly around the site of entry of the needle thus restraining it <u>in situ</u>. The syringe was clamped in position to prevent accidental removal of the needle.

The tracer was delivered slowly so as to prevent spread out of the barbel. The concentrations used were 1% WGA-HRP in YFWTS or 50% HRP in YFWTS.

On completion of delivery, the needle was slowly removed whilst tying the cotton even more tightly so as to prevent leakage of the tracer from the puncture.

Animals were then revived and maintained in aquaria at temperatures ranging from 18-23 °C for a variety of survival times. Table 5 shows the experimental features for all animals in this series of experiments. Subsequent

to sacrifice, procedures were as described in Chapter 2.

Summarizing the details of tracer delivery into a barbel:

1. Anaesthetic.
2. Artificial respiration.
3. Cotton loop around the relevant barbel.
4. Puncture of barbel wall with micropipette.
5. Insertion of syringe needle.
6. Restrain needle in position by tying cotton loop.
7. Gradual tracer delivery.
8. Removal of needle whilst tying loop further.

Table 5

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Barbel (N:nasal) (M:maxillary)	Survival (days)
CF17	157	18.5	1% WGA-HRP	3	N	8
CF18	185	17	" "	3	M	8
CF19	177	18	" "	3	N	8
CF20	118	14.5	" "	3.5	M	8
CF21	296	21.5	" "	4	N	6
CF22	136	16.5	" "	2.5	M	
			" "	2.5	N	6
CF23	187	14.4	1% WGA-HRP	3	N	
			50% HRP	4	M	7
CF24	112	14.5	1% WGA-HRP	3	N	
			50% HRP	4.5	M	7
CF25	115	14.4	1% WGA-HRP	3	M	
			50% HRP	3	N	7
CF26	188	14.3	1% WGA-HRP	3	M	
			50% HRP	3	N	7
CF28	147	16.5	1% WGA-HRP	2	N	
			" "	2	M	17

Table 5 (continued).

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Barbel (N:nasal) (M:maxillary)	Survival (days)
BL01	128	15.9	1% WGA-HRP	3	M	41 hours
BL02	83	14	" "	2	M	48 hours
BL03	121	16.4	" "	2	M	63 hours
BL04	84	14	" "	4	M	62 hours
BL05	162	18	" "	3.5	M	78 hours
BL06	174	18.8	" "	2.5	M	71 hours
BL09	104	15.5	" "	3.5	M	6
BL10	103	16	" "	3.7	M	6
BL11	115	15.8	" "	2.5	M	6
BL12	122	16.2	" "	2.5	M	6

RESULTS

Following histochemical detection of 1% WGA-HRP, reaction product indicative of orthograde transport was observed in ipsilateral regions of the central nervous system. No label was observed in contralateral sites.

In the experiments employing a post-injection survival period of less than 71 hours, inadequate quantities of the tracer had been transported to allow detection, despite the use of a sensitive chromagen. Thus, a minimum period of 3 days survival was necessary following tracer injection and, in practice, survival times of between 6 and 8 days were found to result in the most intense labelling.

Only in two experimental animals (CF18 and CF23) was reaction product observed in cell bodies of brainstem motor neurons, and in these instances the retrograde label was of very low density.

Labelling of cell bodies subsequent to retrograde transport of the tracer tends to be more efficient than labelling of terminals following transganglionic transport. In view of this and the finding that soma were labelled, very lightly, in only two cases, the retrograde label observed was considered artefactual.

The orthograde label was found in two gross areas, the facial lobe and the funicular region of the spinal

cord, and will be considered separately.

Facial lobe label

Orthograde reaction product was confined to different regions of the ipsilateral half of the facial lobe following injections of tracer into the maxillary or nasal barbel.

Injection of 1% WGA-HRP into the maxillary barbel consistently resulted in reaction product distributed within a caudolateral quadrant of the ipsilateral facial lobe (Fig.4.1). This label was not present throughout the complete depth of the lobe but confined to the intermediate levels. These levels are indicated in Figure 4.2, a transverse section through the labelled zone.

Figures 4.3 and 4.4 show two series of adjacent horizontal sections through the facial lobe (CF18 and BL11) outlining the area and volume of the facial lobe where reaction product was observed following tracer injection into the ipsilateral maxillary barbel. The indicated zones can be seen to be of equivalent size and position in both of these examples. They are indicative of the label obtained in the facial lobe following all injections of 1% WGA-HRP into the maxillary barbel.

Figure 4.1.

Horizontal section through the facial lobe following injection of WGA-HRP into a maxillary barbel. Orthograde reaction product (O) can be seen in the caudolateral region of the lobe.
The routes of the primary afferent inputs to the facial lobe from each side (nVII) enter from the rostral aspect of the lobe.
R: rostral. C: caudal. M: midline.
Scale bar: 500μm.

Figure 4.2.

Transverse section through the facial lobe showing the intermediate dorso-ventral level of the label (O) demonstrated in Figure 4.1.
D: dorsal. V: ventral. M: midline.
Scale bar: 500μm.

Figure 4.1

Figure 4.2

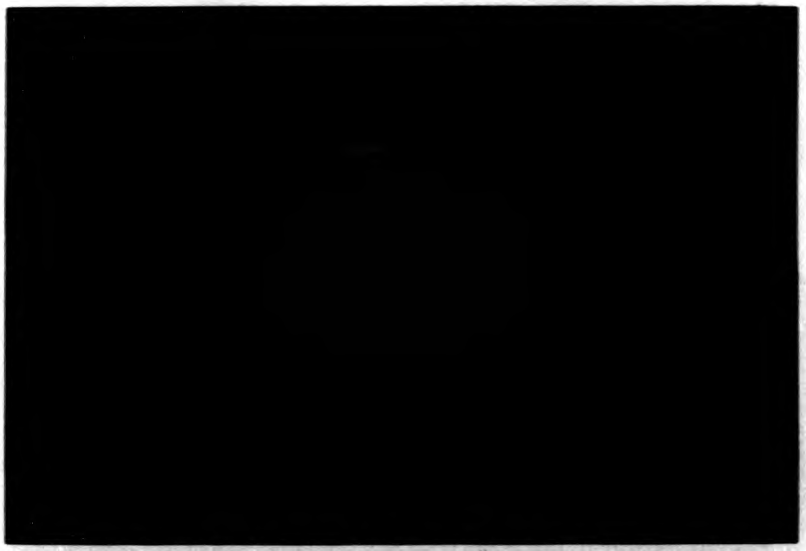
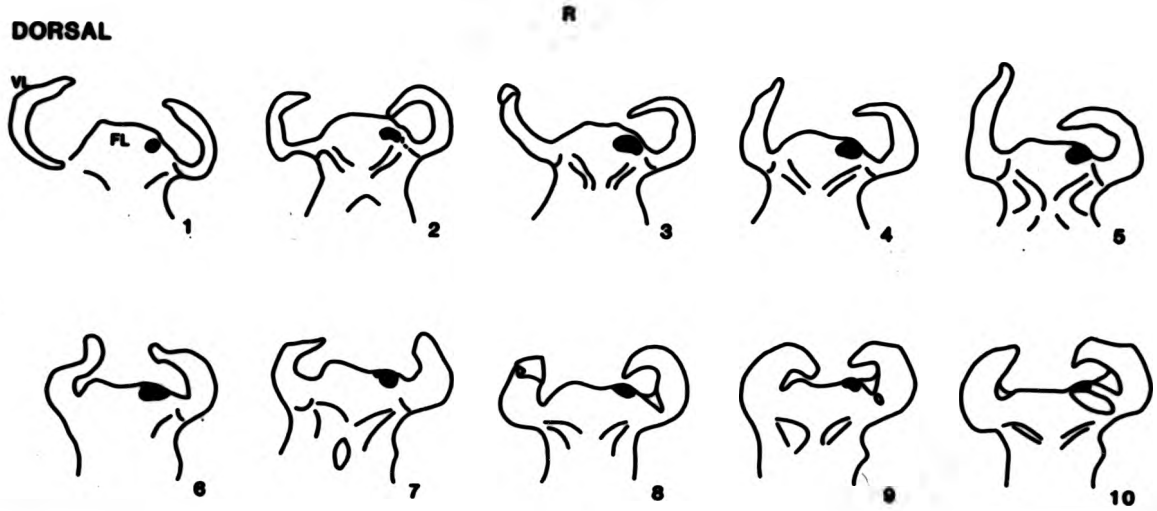


Figure 4.3.

These drawings show the labelled regions (shaded areas) in a series of horizontal sections through the medulla oblongata of CF18 after injection of 1% WGA-HRP into the left maxillary barbel.
Upper left is the most dorsal section displaying label whilst bottom right is the most ventral labelled section.
FL: facial lobe. VL: vagal lobe.
R: rostral. C: caudal.

Figure 4.3

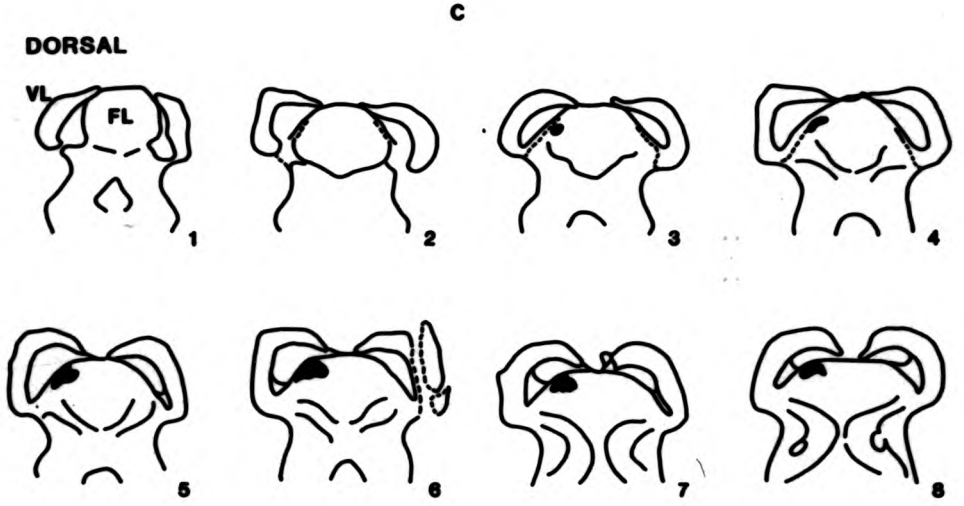


CF18

Figure 4.4.

These drawings show the labelled regions (shaded areas) in a series of horizontal sections following injection of 1% WGA-HRP into the right maxillary barbel of BL11. Conventions as for Figure 4.3.

Figure 4.4



BL11

As a result of injections of 1% WGA-HRP into the nasal barbel, reaction product was distributed within a medial portion of the ipsilateral facial lobe (Fig.4.5). Again, labelled terminals were not found throughout all levels but confined to an intermediate position (Fig.4.6).

The two series of diagrams presented in Figures 4.7 and 4.8 show the distribution of label observed in two animals (CF17 and BL12) following unilateral injection of 1% WGA-HRP into the nasal barbel.

As can be seen, the zone of reaction product tended towards a rostromedial position, in contrast to the caudo-lateral region labelled following delivery of the tracer into the maxillary barbel. It was concluded therefore that the neurons supplying the separate barbels project to separate regions of the facial lobe.

Concerning the tract in which the primary afferent fibres from the barbels course, reaction product was only occasionally observed. In these few instances where it was present (following delivery of 1% WGA-HRP) this reaction product was very diffusely distributed.

The precise relationships of the labelled terminals to the intrinsic cells of the facial lobe were not amenable to analysis at the light microscopic level. No differential affinity for any of the various cell types were apparent at this level.

Figure 4.5.

Horizontal section through the facial lobe following an injection into a nasal barbel. Orthograde reaction product (O) can be seen in the rostromedial region of the lobe. The course of the primary afferent inputs (nVII) to the facial lobe is visible the rostral aspect of the lobe.
R: rostral. C: caudal. M: midline.
Scale bar: 800µm.

Figure 4.6.

Transverse section through the facial lobe showing the intermediate depth of the label (O) shown in Figure 4.5 following injection of 1% WGA-HRP into a nasal barbel.
D: dorsal. V: ventral. M: midline.
Scale bar: 800µm.

Figure 4.5

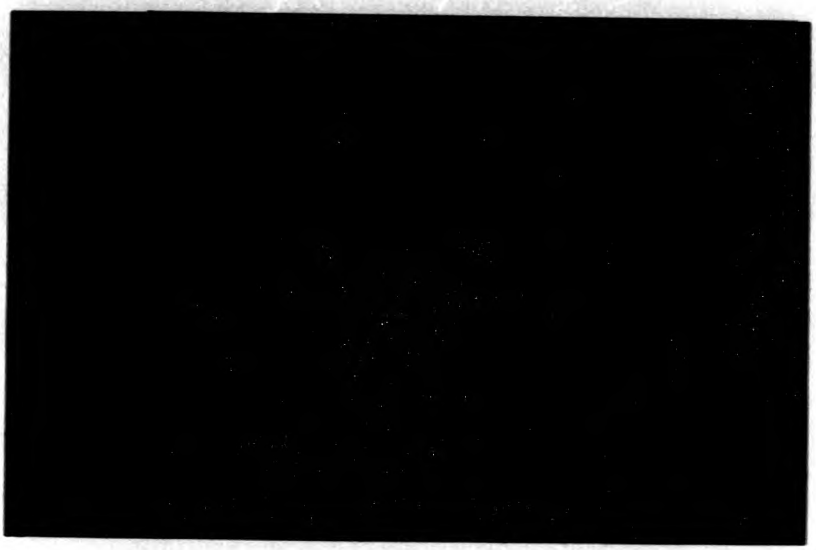


Figure 4.6

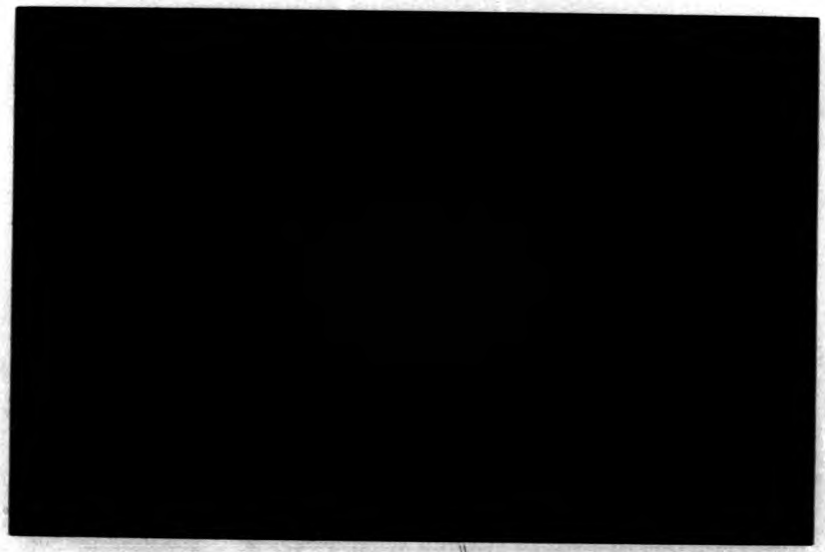


Figure 4.7.

These drawings show the labelled regions (shaded areas) in a series of horizontal sections through the medulla oblongata of CF17 following injection of 1% WGA-HRP into the left nasal barbel.

FL: facial lobe. VL: vagal lobe.

R: rostral. C: caudal.

Figure 4.7

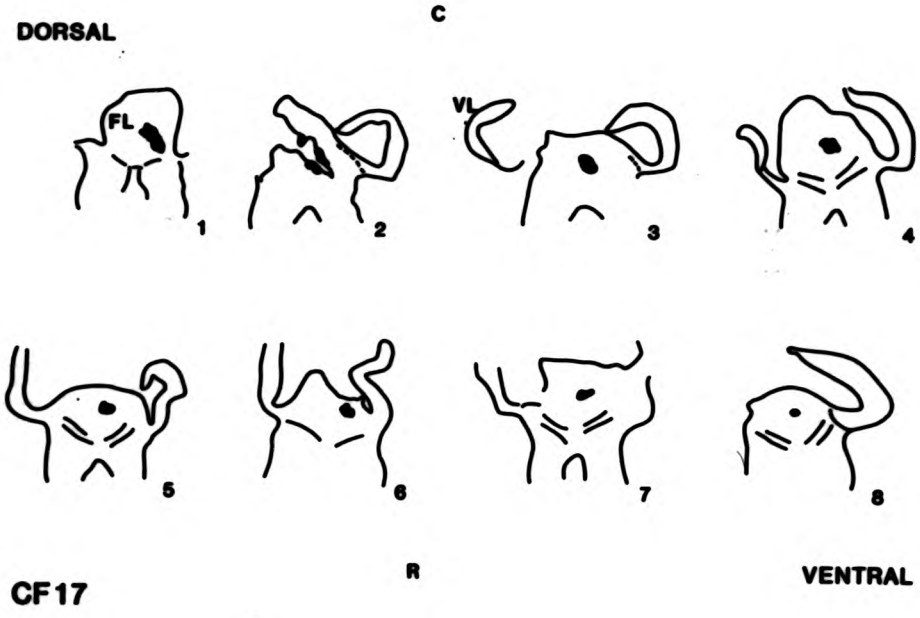
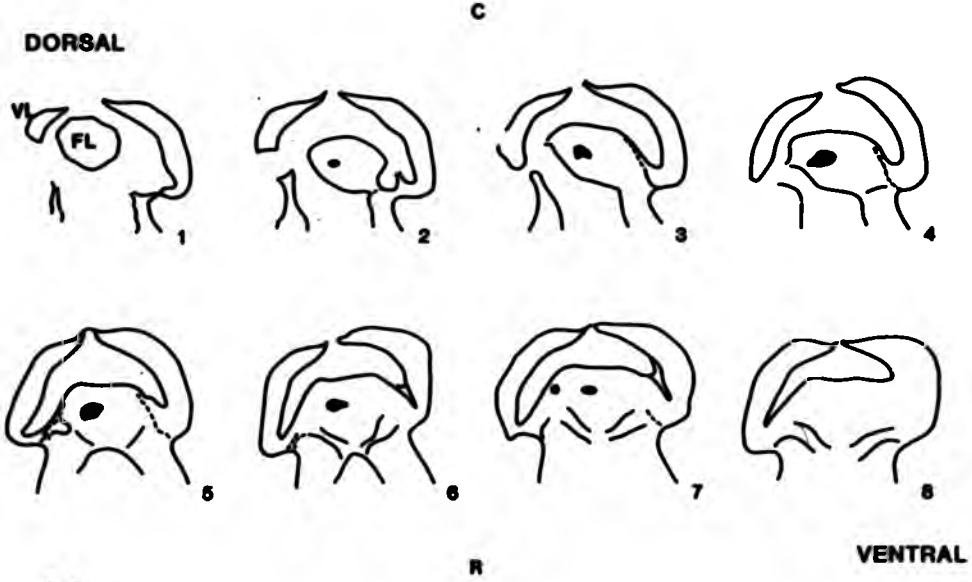


Figure 4.8.

These drawings show the labelled regions (shaded areas) in a series of horizontal sections following injection of 1% WGA-HRP into the right nasal barbel of BL12.
Conventions as for Figure 4.7.

Figure 4.8



BL12

However, orthogradely-transported label was sometimes observed in the form of dense accumulations in the appropriate region rather than scattered uniformly within that region (Fig.4.9). This suggested that the afferent terminals in these instances may be organised in discrete "glomerular" fashion within the topographically appropriate region of the facial lobe.

The two experiments performed using 1% WGA-HRP to bilaterally label the projections of the separate barbels in the same animals (CF22 and CF28) provided no extra data concerning afferent distribution in the facial lobe.

In the case of animal CF22, the terminal projection of the nasal barbel innervation alone was observed. The reasons for the lack of transport following injection of the tracer into the contralateral maxillary barbel were unclear; tracer delivery into the maxillary barbel was, in practice, less problematic than the nasal barbel due to the greater size and diameter of the former.

In the case of animal CF28, neither projection was observed. Assuming successful tracer delivery into the individual barbels, the lengthy post-injection survival period (17 days) allowed in this particular experiment may have been excessive. The transported tracer appears to have undergone degradation within the axonal endings (c.f. Droz and DiGiamberardino 1973).

Figure 4.9.

Horizontal section through the facial lobe showing the non-uniform accumulation of orthogradely-transported WGA-HRP (O) following injection into the ipsilateral nasal barbel.

Scale bar: 250μm.

Figure 4.9

Funicular label

In addition to that observed in the facial lobe, injections of 1% WGA-HRP into the barbels resulted in orthograde label in the ipsilateral medial funicular nucleus.

Delivery of the tracer into the maxillary barbel produced dense reaction product in the medial portion of this region (Fig.4.10). In contrast ejection into the nasal barbel resulted in a small labelled zone within the lateral extent of the nucleus (Fig.4.11).

So, as observed in the facial lobe projections of neurons innervating the barbels, the connections of the medial funicular nucleus also exhibited a topographical organisation, the maxillary barbel being represented in the medial region and the nasal barbel in the lateral region.

Comparison of tracers

Throughout this series of experiments, injections into the maxillary barbel produced reaction product within similar limits of the caudolateral quadrant of the facial lobe (compare CF18 and BL11). In addition, the individual injections of 1% WGA-HRP into the nasal barbel resulted in similar extents of the rostromedial region being labelled. It was concluded, therefore, that the spread of tracer in the individual barbels was equally limited confirming the

Figure 4.10.

Horizontal section through the region of the rostral spinal cord and caudal medulla. Orthograde reaction product (O) is visible in the medial region of the medial funicular nucleus following injection of 1% WGA-HRP into the ipsilateral maxillary barbel.
R: rostral. C: caudal.
Scale bar: 200μm.

Figure 4.11.

Horizontal section through the region of the rostral spinal cord and caudal medulla. Orthograde reaction product (O) following injection of 1% WGA-HRP into the ipsilateral nasal barbel can be seen in a more lateral position than that shown in Figure 4.10.
R: rostral. C: caudal.
Scale bar: 200μm.

Figure 4.10

Figure 4.11

efficiency and consistency of the injection procedure.

This was a necessary proviso for the empirical comparison of the transganglionic transport of HRP and WGA-HRP in the facial nerve pathways under investigation. With respect to this comparison, the greater effiency of the conjugate was confirmed.

The reaction product was sufficiently optically dense for light microscopy in only one of the four experiments (CF24) utilising unconjugated HRP. Comparison of the resultant label in the caudolateral quadrant of the facial lobe following this injection of 50% HRP into the maxillary barbel with that following delivery of 1% WGA-HRP (BL18) (Figs.4.12 & 4.13) shows the difference in intensity of reaction product. The volume of tracer delivered in each case was comparable but the reaction product of the transported conjugate was much denser than that of transported HRP.

No reaction product was observed in the ipsilateral facial lobe following injection of 50% HRP into a nasal barbel (CF25 and CF26). This contrasts with its consistent detection following delivery of 1% WGA-HRP.

In view of the observed transport of both tracers in the neurons supplying the maxillary barbel, and assuming no differences between the barbels in the physio-

Figure 4.12.

Horizontal section through the facial lobe showing orthograde reaction product (O) in the left caudolateral region following an injection of 50% HRP into the left maxillary barbel.
R: rostral. C: caudal.
Scale bar: 500µm.

Figure 4.13.

Horizontal section through the facial lobe showing orthograde reaction product (O) in the right caudolateral region following an injection of 1% WGA-HRP into the right maxillary barbel. Note the presence of greater quantities of reaction product in this case compared with that shown in Figure 4.12.
R: rostral. C: caudal.
Scale bar: 500µm.

Figure 4.12

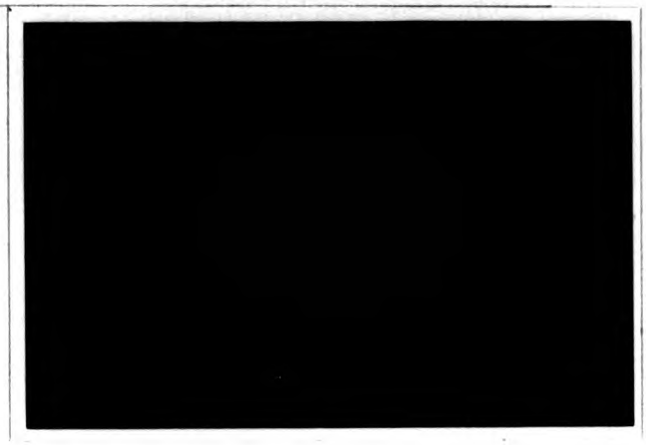
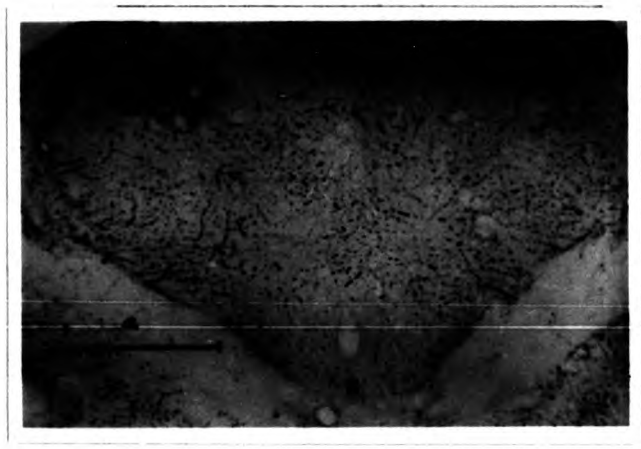


Figure 4.13



logical mechanisms underlying uptake, it must be presumed that insufficient HRP was transported from the nasal barbel to allow its detection. This occurred despite delivery of a fifty-fold greater concentration of tracer.

No label was observed in the funicular region following injections of unconjugated HRP into the barbels. This was the case even in the sole experiment which resulted in reaction product in the facial lobe.

DISCUSSION

This study on the central projections of neurons innervating the barbels has confirmed the advantage of utilising WGA-HRP as a transganglionic tracer substance in the fish nervous system.

The data also allow some quantifiable estimate of the advantage of the lectin conjugate. The injection of equal volumes of HRP and WGA-HRP into the nasal barbel gave rise to detectable reaction product in the case of the latter only.

As the concentration of the unconjugated HRP was fifty times greater than that of the WGA-HRP, it appears that the conjugate must be at least fifty times more efficient. This compares favourably with a forty-fold figure reported previously for a mammalian system (Trojanowski, Gonatas and Gonatas 1981).

Whether this added potency was due to enhanced uptake by the neuronal processes ramifying in the superficial layers of the barbels or to more vigorous intra-axonal transport mechanisms cannot be ascertained from these experiments. However, previous studies addressing this issue have indicated the presence of an active endocytosis of the conjugate, in contrast to a general pinocytotic uptake of unconjugated extracellular HRP (see Chapter 1). The crucial difference between the efficacies of the two tracers appears, therefore, to lie

in their differential internalisation by neurons.

Concerning the anatomical data provided by the use of WGA-HRP in the facial system of the carp, this present study is not directly comparable with the classical studies of Herrick (1905) and Barnard (1936) on the cyto-architectural organisation of the facial lobe.

These details are not readily recoverable from a tracer study, whilst aspects of topographical representation are not definitively presented in studies using normal material.

One feature described by Herrick (1905) which may be applicable to this study was the apparent presence of "rosettes" of small neurons in the centre of the cyprinoid facial lobe. This anatomical arrangement may coincide with the glomerular-like organisation of endings sometimes visualised in this study of afferents to the facial lobe. If this is the case, it clearly suggests that the primary afferent neurons supplying the barbel taste buds may project onto these small perikarya. This would be in agreement with a previous speculation that facial nerve terminals synapse with the small cells of the facial lobe (Yasgashi 1973).

As regards other experimental anatomical studies on the projection of the facial nerve and its branches to the central nervous system, the present report differs from

the previous analyses on a number of points, at least as far as the facial lobe connections are concerned.

Using a degeneration technique, unilateral sectioning of the facial nerve close to the brain has shown argyrophilic debris throughout the ipsilateral half of the facial lobe (Luiten 1975). In addition, a small amount of terminal degeneration was observed in the contralateral half of the lobe as well as in the glossopharyngeal lobe and its border with the vagal lobe.

Similarly, degenerating neural elements were observed in both the ipsilateral and contralateral facial lobe following unilateral facial nerve section in the Crucian carp (Morita, Ito and Masai 1986).

The tracer study reported here contrasts with these results in three ways:

1. Label was not found throughout the ipsilateral portion of the facial lobe, but was confined to limited regions.
2. No label was observed in the contralateral half of the facial lobe.
3. Both the glossopharyngeal and vagal lobes were devoid of transported tracer.

Several explanations of these differences are possible. Firstly, the occurrence of degeneration in all regions of the ipsilateral facial lobe following facial nerve rhizotomy adjacent to the brain is not surprising. The use of this approach would sever all the branches of

the nerve proximal to their ganglionic cell bodies.

Thus the major, if not entire, primary afferent input to the facial lobe would be interrupted with the consequent anterograde degeneration of fibres projecting to any and all regions of the lobe. Clearly, no details of topographic separation of innervation patterns within the facial lobe are definitively available using this approach.

In contrast, the current study traces the connections of branches within the nerve innervating individual barbels, therefore allowing topographic analyses to be made.

Secondly, the occurrence of degeneration in the contralateral half of the lobe, in contrast to the present findings, could be explained as a consequence of the tract tracing method used.

In sensory systems such as the visual and olfactory modalities, massive deafferentation on a scale equivalent to the complete severing of the facial nerve, has previously been shown to induce trans-synaptic anterograde atrophy of secondary connections (Cowan 1970; Carlsen, de Olmos and Heimer 1982).

It could be suggested therefore that this process may underlie the observation of degenerating neurons in the contralateral facial lobe.

Nevertheless, it may be more parsimonious to view

this observation as due to the lesioning of a branch of the facial nerve which does in fact project, primarily, to the contralateral side. This branch, however, would evidently not be involved in the innervation of the barbels as it was not observed in the current studies.

Comparative support for this view comes from an investigation of barbel nerve projections in siluroid fishes (Finger 1976). This study also found no evidence for a contralateral projection from the barbel afferents. This is made more significant by the fact that a degeneration technique was utilised to trace the central connections from ganglion cell bodies associated with a particular barbel.

However, it should be mentioned that siluroids and cyprinoids are not directly comparable. In cyprinoids, the facial lobe is a midline fused structure whereas in the siluroid circumstance the facial system is represented, in many cases, in bilateral un-fused lobes.

The destruction of branches unrelated to barbel innervation incurred by rhizotomy of the facial nerve can also explain the third difference highlighted.

The observation of facial nerve afferent inputs to the ipsilateral glossopharyngeal and anterior vagal lobe in normal material (Herrick 1985) implies that the atrophic elements seen in these sites after facial nerve section may not be due to trans-synaptic degeneration.

The presence of these projections would infer that some branch or branches of the facial system unrelated to barbel innervation, as defined by the present study, may be reflective of their role within the functions co-ordinated by the glossopharyngeal and vagal lobes.

In this respect, it is important to note that facial nerve components also supply taste buds in the anterior part of the mouth. This pattern of innervation may well overlap with that of the glossopharyngeus and vagus nerves.

In view of the known role of these latter lobes in pharyngeal mechanisms, an association with a part of the facial system supplying the buccal region would not be wholly unexpected.

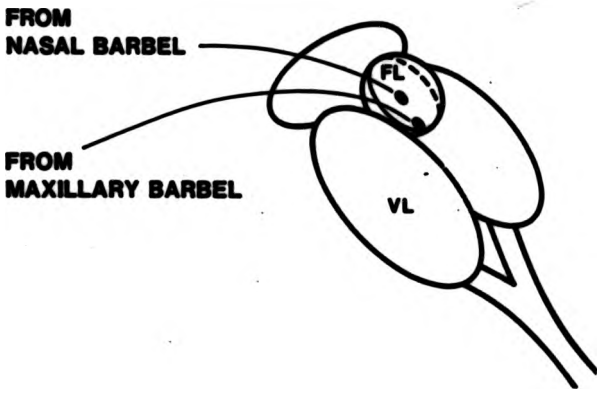
As regards the presence of a gustatory somatotopy within the facial lobe of the carp, the present study indicates a distinct separation of the terminal projection zones associated with the nasal and maxillary barbels. The primary afferent neurons supplying the maxillary barbel have been shown to terminate in a caudolateral region of the facial lobe at an intermediate depth whereas the primary afferents innervating the nasal barbel project to a more rostromedial location in the lobe. Figure 4.14 provides a summary diagram of this organisation.

Such a somatotopy has also been observed using electrophysiological procedures in both the carp (Marui

Figure 4.14.

This diagram illustrates the somatotopic organisation of the projections of the barbel innervation in the facial lobe. The maxillary barbel is represented in the caudolateral region whilst the nasal barbel is represented at a more rostral and medial location.
FL: facial lobe. VL: vagal lobe.

Figure 4.14



1977) and another cyprinoid, the goldfish (Peterson 1972), although the latter study was concerned with tactile responses only.

The main conclusion from the electrophysiological results of Marui suggests that the axis of somatotopy is such that the anterior-posterior body axis is represented in the ventral-dorsal lobe axis. This contrasts with the goldfish in which the anterior-posterior body axis is represented in the anterior-posterior lobe axis.

The results of the current tracer study do not allow a determination of the axis of somatotopy. In order to be able to provide an anatomical basis, details of the pattern of projection to the facial lobe from another branch of the facial nerve, preferably the recurrent branch innervating flank taste buds, are necessary. In this way a third "reference point" would be provided whereby the axis of gustatory somatotopy could be more accurately plotted.

It is clear from this tracer study, however, that the regions of the carp facial lobe in which the primary afferents from the individual barbels distribute are proportionately greater in volume relative to the area of the body surface which they represent. A similar expanded facial lobe representation of barbels has been reported in anatomical (Finger 1976) and electrophysiological studies (Marui and Caprio 1982) of Ictalurus.

The barbels provide an increased surface area with

which to sample the environmental substrate for nutrient material and therefore have a significant role in localising such material. Also, the barbels possess greater densities of taste buds than, for example, the flanks. Their disproportionate representation in the facial lobe(s) therefore probably reflects this increased density of receptors.

The precise limits of the barbel representation were not available from the electrophysiological study of the carp. The anatomical evidence shows a distinct separation of their representations in the ipsilateral half of the facial lobe. The relatively large degree of separation observed in the current studies might be thought surprising in view of the close proximity of the two barbels. However, as the barbels lie either side of the lateral extents of the upper lip and as the lips are also densely populated with taste buds it might be expected that this region would also be disproportionately represented in the facial lobe.

The results presented here are in general agreement with a recently published report of the central projections of individual branches of the facial nerve in Cyprinus carpio (Kiyohara, Shiratani and Yamashita 1985).

These authors applied HRP to the cut distal end of individual branches and observed a topographical representation for each branch within the ipsilateral

facial lobe. In addition, using an electrophysiological approach, they determined the peripheral distribution of these branches. The receptive fields for each major branch innervating the barbel region overlapped considerably. Thus, from their data it does not appear possible to assign particular facial nerve branches to individual barbels. It is not possible, therefore, to directly compare the topographical map provided from that study with the map obtained using the approach utilised in the investigations reported here. However, it can be stated that the two studies do not appear to conflict with each other.

Comparing the charts of labelled regions obtained from the two different approaches:

1. The facial lobe label obtained after tracer injection into the maxillary barbel coincides with facial lobe label following cut nerve application of HRP to only the ramus maxillaris and the ramus palatinus. It seems, therefore, that of the branches investigated, only these two supply the maxillary barbel.
2. The ramus mandibularis supplies neither the maxillary nor the nasal barbel.
3. The nasal barbel receives no significant supply from any of the facial nerve branches investigated.

In addition to the somatotopic distribution of barbel afferents within the ipsilateral facial lobe, the

present studies have shown that the inputs to the medial funicular nucleus are topographically organised. The innervation of the larger, maxillary branch includes a component which projects to a medial region within the nucleus whereas the nasal barbel receives a component which terminates in a lateral portion of the nucleus.

The projections to this cervical spinal zone have previously been described using a degeneration procedure (Luiten 1975), but for reasons already described, no details of topography were extracted from that study.

The functional significance of this projection to the medial funicular nucleus is unclear. However, in addition to taste buds, the barbel epithelium also contains free nerve endings (Herrick 1985) and it has been generally believed that the taste buds receive input solely from the facial nerve whilst the free nerve endings are of trigeminal origin.

The funicular nuclei have previously been suggested to be a correlation centre for tactile stimulation conveyed by trigeminal components from somatic sources (Herrick 1987). Thus, it may be the case that the projection to the funicular region is a trigeminal component of the barbel innervation and conveys tactile information from the free nerve endings. Tactile responses have been recorded in the facial lobe following mechanical stimulation of the lips and other regions of the body

surface in cyprinoids (Peterson 1972; Marui and Funakoshi 1979; Funakoshi, Kawakita and Marui 1981) and in Ictalurus (Marui and Caprio 1982), but there appear to be no reports of similar investigations of the medial funicular nucleus.

Alternatively, the projection from the barbels to the medial funicular nucleus may represent the neural substrate of an additional chemosensory system.

In addition to the gustatory modality, in which taste buds possess receptor cells innervated by facial, glossopharyngeal or vagal nerves, there exists also the common chemical sense. This "modality" is often thought to be mediated via free nerve endings (Parker 1922) and is innervated by general somatic afferent (e.g. spinal or trigeminal nerves) nerves (Silver and Finger 1984).

In the sea robin, Prionotus carolinus, the common chemical sense has been assumed to be particularly well developed. In this species, the first three fin rays of the pectoral fin are separated from the rest of the fin and are used for exploring the environmental substrate. The significant features have been that taste buds are absent but these fin rays possess a chemosensitive capacity. Also, the innervation of the free fin rays derives entirely from spinal nerves (Finger 1982).

As to its function, the common chemical sense was originally viewed as having a nociceptive role (Parker 1922), allowing the animal to respond to harmful substances. However, electrophysiological studies of the

spinal nerve innervation of the free fin rays in Prionotus have shown responses to relatively low concentrations of amino acids which are not aversive and have also been shown to be gustatory stimuli (Silver and Finger 1984). On the basis of this evidence therefore, it would appear that the common chemical sense cannot be generally thought of as having a nociceptive function. There appears to be, however, some dispute as to the identity of the receptor responsible for the chemosensitivity of the free fin rays in Prionotus and it may therefore be the case that this chemosensitivity cannot be ascribed to the common chemical sense. In fact, it has been suggested that sparsely distributed chemoreceptor cells may underlie this sense rather than free nerve endings (Lane and Whitear 1982). It seems therefore that, not only is the function of the barbel projection to the medial funicular nucleus unclear, but in addition, if it does represent the primary afferent projection of the common chemical sense, it may not originate from free nerve endings.

In addition to the suggestion that the medial funicular nucleus receives tactile information originating from free nerve endings, there is evidence, as previously stated, for a tactile input to the facial lobe. The identity of the receptor responsible for this tactile information is not definitively known. However, it has been suggested from electrophysiological studies in the

puffer fish, Fugu pardalis, that gustatory and tactile activity are transmitted to the facial lobe by the facial and trigeminal nerve respectively (Kiyohara, Hidaka and Tamura 1975). A similar situation might exist in the carp (Marui and Funakoshi 1979). It may be the case therefore (akin to the suggestion for the projection of the barbel innervation to the medial funicular nucleus), that the free nerve endings in the barbel epithelium are the receptors for tactile stimuli and that they project to the facial lobe in a somatotopic manner which mirrors that from the taste receptors. The tactile and gustatory representations in the facial lobe have previously been observed to be coincident (Marui 1977).

In contrast, electrophysiological studies in Ictalurus convincingly indicate that receptors innervated by the facial nerve can be both chemo- and mechano-sensitive (Davenport and Caprio 1982). In this case, if the classical view that taste buds are supplied by the facial nerve and free nerve endings originate from trigeminal or spinal nerves is correct, taste buds may have a tactile role in addition to a gustatory function. Perhaps accessory cells within the taste bud complex (Reutter 1978) might have a mechanosensory capacity. On the other hand, an alternative view might be that free nerve endings provide the tactile information and are formed from branches of facial nerves innervating neighbouring taste buds. Certainly, in either of these

cases, the somatotopic organisation of the facial lobe would be expected to incorporate overlapping gustatory and tactile representations.

Much of the literature is contradictory or inconclusive regarding barbel receptors and seems to stem from a lack of data concerning the free nerve endings. In order to begin clarifying some of the issues discussed above, it is important to document the receptor types which are represented in the facial lobe and medial funicular nucleus. For example, do the free nerve endings project to the medial funicular nucleus? Also, do free nerve endings and the taste bud innervation both project to the facial lobe or is the facial lobe representation derived solely from the taste bud supply?

With regard to these questions it would be interesting to deliver WGA-HRP into these structures and trace their peripheral relationships, a method which has proved successful for neurons projecting to the vagal lobes from palatal receptors (Chapter 6).

In summary, from a technical viewpoint, the results reported here have confirmed conclusions from the previous chapter that the WGA-HRP conjugate is more efficient than unconjugated HRP for tracing axonal projections in the teleost nervous system. This particular study has allowed

quantification of this superiority such that WGA-HRP seems to be at least fifty times more efficient. This may be due to internalisation of greater quantities of the tracer into the neurons as a result of specific receptors for the lectin component of the tracer.

The anatomical details provided by this investigation show that the sensory neurons supplying the individual barbels project solely to ipsilateral central nervous sites. Each barbel is represented in both the facial lobe and the medial funicular nucleus. It has been suggested that these may indicate separate central relay sites for distinct functional systems.

The inputs to both central sites have been shown to be topographically organised thus maintaining a somatotopy of the barbel representation in the primary relay nuclei.

CHAPTER 5

CENTRAL CONNECTIONS OF NEURONS INNERVATING THE HYOID PROTRUSION

INTRODUCTION

The lining of the buccopharyngeal cavity in *Cyprinus carpio* is well populated by taste buds. Although these receptors are found throughout, they are found more densely in particular regions within the cavity. Their presence on the palatal organ has previously been referred to in Chapter 3. There are also dense accumulations on the gill arches and gill rakers (Herrick 1905). In addition, they are also found within the epithelial lining of a small muscular region localised around the midline on the floor of the pharyngeal cavity (see initial Introduction).

No literature appears to describe this mid-ventral hyoid structure and no specific nomenclature is available. Thus, for the present purposes, it is referred to as the "hyoid protrusion".

The structure of this hyoid protrusion resembles, in essence, that of the palatal organ. That is, its epithelial layer is populated with taste buds and the underlying subepithelial tissue contains a matrix of striated muscle fibres.

Although this protrusion in the carp does not

appear to be described in detail in the literature, taste buds in a similar region have been described for other species of fishes. For example, mid-ventral pharyngeal taste buds have been described in Ameirus melas (Landacre 1907), and in the silverside Menidia (Herrick 1899).

The taste buds of this region in Ameirus are innervated by the ventral post-trematic branch of the glossopharyngeal nerve (Landacre 1907). The same is true for Menidia, and this ventral branch of the glossopharyngeal nerve has been described as "the true lingual nerve" (Herrick: in a note appended to Landacre 1907). In view of this innervation it may well be pertinent to refer to the hyoid protrusion as the "tongue". However, it is clearly not a tongue in the generally accepted sense.

The glossopharyngeal nerve in cyprinoids projects onto a small tuberosity found between the medial vagal lobe and lateral facial lobe (Herrick 1905 - see initial Introduction). This is known as the glossopharyngeal lobe. It has a laminar structure but is not laminated to the same degree as observed in the vagal lobes and has been described as possessing a medial afferent zone and a lateral efferent zone (Barnard 1936).

The purpose of the present experiments was to trace the central projections of the neurons innervating the taste buds on the hyoid protrusion. The data from other species would predict that these taste buds are innervated by the glossopharyngeal nerve and are therefore represent-

ed in the CNS of the common carp within the glossopharyngeal lobe.

In addition, although the taste buds of this region and their innervation have been described in other species, there appears to be no mention of the muscle fibres which are observed within this zone in Cyprinus carpio.

Thus, a further purpose of these experiments was to trace the source of the motor neurons innervating these muscle fibres.

Finally, surfaces opposing the palatal organ are as involved in the process of sifting and sorting as the palatal organ itself. The hyoid protrusion is situated opposite the rostral portion of the palatal organ. In view of the likely relationship between the two structures during feeding it might be expected to find a close relationship between the central nervous representations of the two structures.

MATERIALS AND METHODS

This series of experiments involved the delivery of tracer into the small hyoid protrusion on the floor of the mouth. The structure is of a relatively small size and is found in a position traversing the midline. Thus the spread of tracer could not be definitively limited to unilateral sites as was the case for injections into the palatal organ or into the barbels. Also, injections into the core of the structure were practically difficult. This was due to its propensity for deformation by the tip of the syringe needle rather than penetration. In addition, it was necessary to approach the structure through the open mouth. As the illumination was also directed through the oral opening, optimum lighting for tracer delivery was sometimes compromised. Nevertheless, a number of conclusions regarding the innervation of this region were possible.

Eight animals were used in this study. Following initial anaesthesia (ethyl m-amino benzoate, 1:5000 in water) the animals were weighed and measured for length (snout to base of caudal fin). They were loosely wrapped in damp tissue and placed on the fish box.

Approaching through the open mouth, the surface of the hyoid protrusion was punctured with the needle (33 gauge) of a tracer-filled 5µL Hamilton syringe. This generally required several attempts due to the lack of

rigidity of the structure.

After successful insertion, the tracer (1% WGA-HRP in teleost saline) was ejected. Unfortunately, ejection of even the smallest volume of tracer sometimes resulted in an immediately observable leakage back along the track of the needle. Therefore, the values given in Table 6 for the individual volumes delivered necessarily refer only to that ejected from the syringe. A precise quantification of the volume delivered to the site of innervation was not always possible.

The needle was maintained in position for up to 10 minutes to minimise the leakage and the animals were then revived. They were maintained, prior to sacrifice, in aquaria at 18-22 °C. All subsequent procedures were as described in Chapter 2. Table 6 indicates the experimental details of this study.

As can be seen from Table 6, relatively lengthy survival times were generally allowed in this study. This was regarded as necessary following the initial experiment (4 day survival) and because of the comparatively long distance for axonal transport of the tracer from the floor of the mouth to the hindbrain.

Table 6

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (µL)	Survival (days)
PL17	91	14.5	1% WGA-HRP	4.5	4
PL21	95	14.8	" "	4	9
PL22	100	15.2	" "	4.5	9
PL23	92	15	" "	3.5	12
PL24	91	14	" "	2.5	12
PL26	120	15.7	" "	2.5	11
PL33	109	15.8	" "	2	8
PL34	124	16	" "	2	8

Summarizing the details of tracer delivery:

1. Anaesthesia.
2. Mouth held open.
3. Insertion of syringe needle into the hyoid structure.
4. Gradual tracer delivery.
5. Revival.

RESULTS

The problems associated with the delivery of the tracer were reflected in the variations in the density of labelling observed amongst the different animals in this series. Nevertheless, considering the series *in toto*, both orthograde and retrograde label were observed in the medulla oblongata.

Orthograde label

The reaction product typical of orthogradely-transported WGA-HRP was found bilaterally in the medulla oblongata.

This occurrence of label bilaterally, however, did not necessarily reflect a bilateral innervation of the hyoid protrusion. The difficulties noted previously regarding the injection procedure prevented the definitive unilateral placement of the tracer. Consequently, the bilateral extent of the reaction product may well have resulted from bilateral spread of WGA-HRP in the structure.

The orthograde label was observed at a location where the rostral limits of the vagal lobe and the rostro-lateral limits of the glossopharyngeal lobe merge. In addition, it was only found in the most ventral sites (Fig.5.1 and Fig.5.2). No strict border could be drawn

Figure 5.1

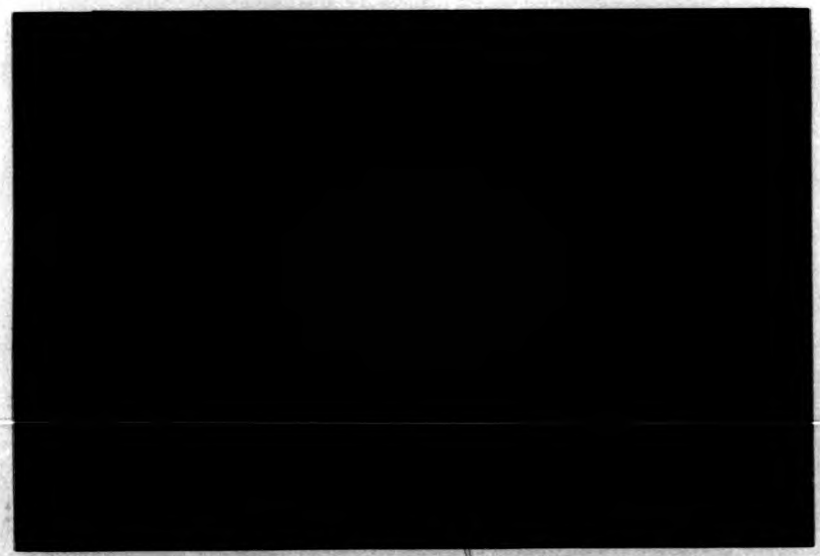
This photomicrograph shows the orthogradely-transported WGA-HRP (O) present in the rostral medulla oblongata following injection into the hyoid protrusion.
Scale bar: 100μm.

Figure 5.2

A horizontal section through the ventral medulla oblongata showing the glossopharyngeal lobe (GL) with the adjacent anterior vagal lobe (VL) and lateral facial lobe (FL). The orthogradely-transported label (O) is present in the anterior and lateral region of the lobe at the border with the vagal lobe.
Scale bar: 100μm.

Figure 5.1

Figure 5.2



between the two lobes as the cytoarchitecture of the region exhibits no distinct boundary in the change from the laminar organisation of the vagal lobes. It was possible therefore, that the labelled region included both glossopharyngeal and vagal components. It was never observed in particularly dense accumulations within this zone as compared with similar reaction product observed in the vagal and facial lobes following palatal or barbel injections, respectively.

This possibly infers a sparse distribution of sensory receptors on the hyoid protrusion. However, this lack of dense labelling could also have been due to the delivery and subsequent neuronal uptake of inadequate quantities of WGA-HRP for the detection of transganglionic transport.

The ramification of this afferent projection was confined to a superficial region (Figs.5.1 and 5.2). Unlike the superficial afferent projection from the palatal organ to the vagal lobe, however, no regular variation in the density of terminals was observed.

Terminal label only was present. No label could be seen within the course of the afferents into this region, either in the glossopharyngeal or vagus roots.

Retrograde label

Retrogradely-labelled motor neurons were observed bilaterally but this labelling, as suggested for the

orthograde label, did not necessarily infer bilateral innervation.

The numbers of labelled motor neurons were never very large. In the case of the primary afferent innervation the paucity of label could be construed as inefficient tracer delivery. The retrograde transport of label to the motor neuron origins, in contrast, is not subject to the need for transganglionic passage. Therefore, in general, the delivery of small volumes of tracer should be adequate to detect its subsequent retrograde transport.

Thus, the presence of limited numbers of labelled motor neurons following WGA-HRP injections suggests a sparse motor innervation of the hyoid structure. This might not be unexpected considering the limited musculature within the structure as compared with the palatal organ.

Concerning the distribution of the labelled cells, they were observed in positions at the bases of the glossopharyngeal lobes and the rostral end of the vagal lobes (Fig.5.3 and Fig.5.4).

No clear structural boundary was apparent between the glossopharyngeal and ipsilateral vagal lobe and their motor nuclei appeared to be continuous with each other. This also appears to be the case in another cyprinoid, the tench (Ariens Kappers, Huber and Crosby 1936).

The pattern of labelling was inconsistent between

Figure 5.3

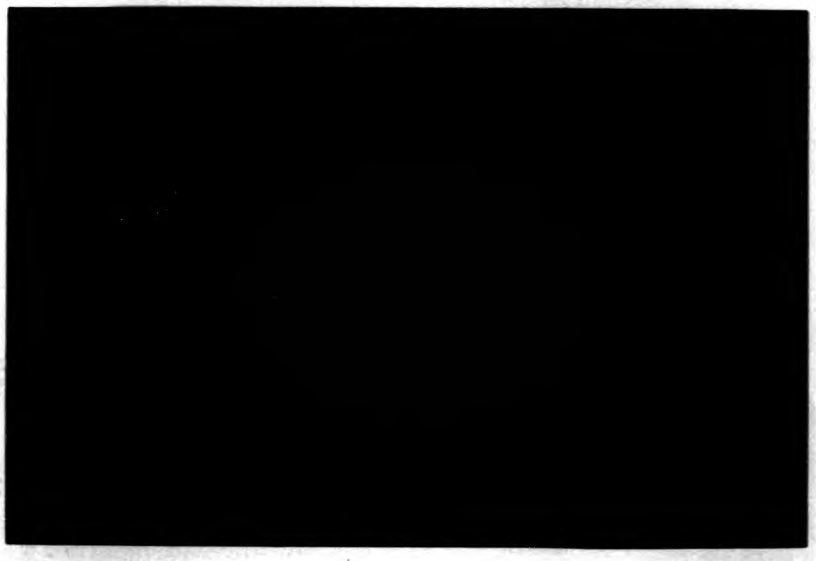
This horizontal section through the ventral medulla shows retrogradely-labelled cells observed following injection of WGA-HRP into the hyoid protrusion.
The cells are located within the glossopharyngeal (G) and anterior vagal (V) motor divisions.
Scale bar: 500μm.

Figure 5.4

This lower power photomicrograph at approximately the same dorso-ventral level as Figure 5.3 shows the location of the retrogradely-labelled cells in relation to the vagal lobe (VL).
The cells can be clearly seen to incorporate glosso-pharyngeal (G) and anterior vagal (V) motor components.
Scale bar: 500μm.

Figure 5.3

Figure 5.4



animals, but overall the labelled cells were observed in a diffuse "sheet" within the glossopharyngeal and anterior vagal divisions of this motor column, interspersed amongst unlabelled perikarya.

These results have been presented as a generalisation of the data obtained from all eight of the animals in the series.

This was regarded as necessary because retrogradely labelled cells were observed in seven of the eight experimental animals whereas reaction product characteristic of orthograde transport was detected in only five of the animals. In addition, the density of label varied between animals depending on the post-injection survival time.

A four day survival period (PL17) resulted in moderately dense reaction products in both the retrogradely and orthogradely-labelled sites outlined above. In contrast, of the animals allowed to survive for 9 days prior to sacrifice (PL21 and PL22), one exhibited no label (PL21) whilst the other showed both low density orthograde and retrograde label (PL22).

In the case of PL21, the histochemical reaction was adjudged to have proceeded as normal due to the occurrence of background endogenous peroxidase activity. Thus, the lack of "experimental" label was not the result of an inefficient detection procedure. In support of this

conclusion, identical reagents were used for the simultaneous treatment of animal PL22 in which label was observed.

Comparing the results following these four day and nine day survival periods, it could have been assumed that the different densities of reaction products were the consequence of an excessive survival time in the latter instances.

However, the densest orthograde and retrograde labels observed within this series of experiments were those following the lengthier survival periods of 11 and 12 days (PL23, PL24 and PL26). In these cases, motor neuron soma (Fig.5.3 and Fig.5.4) and terminal projections (Fig.5.1 and Fig.5.2) were clearly detectable.

Finally, animals which survived for 8 days prior to sacrifice (PL33 and PL34) were found to exhibit the retrogradely-labelled motor neuron soma only. No orthograde label was observed in those sites identified in animals PL17, PL22, PL23, PL24 and PL26.

These results can be summarised in relation to the post-injection survival time. Both orthogardely- and retrogradely-transported tracer were detectable at moderate density in the hindbrain following a four day survival. After eight days post-injection survival, only the reaction product characteristic of retrogradely-transported WGA-HRP was observed. A period of survival of nine days allowed the detection of both retrograde and

orthograde reaction product. Finally, the longest survival periods (eleven or twelve days) resulted in the densest orthograde and retograde reaction products observed amongst all animals. Only one animal (nine day survival) failed to demonstrate either form of transported tracer.

DISCUSSION

These studies have revealed both sensory and motor components of the innervation of the hyoid protrusion within the glossopharyngeal and anterior vagal division of of the medulla oblongata.

The glossopharyngeal lobes are small tuberosities situated medially of the vagal lobes and laterally of the midline facial lobe. The name derives from the observation that it receives the central branches of the glossopharyngeal nerve (Herrick 1985).

The vagal and glossopharyngeal lobes merge with each other such that no specific boundary between them is particularly apparent. This may be a reflection of an overlap of the glossopharyngeal and vagal roots within the region which divides them. The nerve roots have previously been described as confused within this region (Herrick 1985; Barnard 1936).

The sensory afferents from hyoid receptors have been shown in the studies reported here to ramify within the superficial level at the border between the glossopharyngeal and the vagal lobe. Sensory afferents have been observed at a similar superficial level in the vagal lobe following tracer injection into the palatal organ. The organisation of both the vagal and glossopharyngeal lobes may therefore be analogous. However, the structure of the glossopharyngeal lobe is not clearly laminated

throughout as is characteristic of the vagal lobe.

A study of the glossopharyngeal nerve of the crucian carp (Carassius carassius) has suggested the presence of two subroots within the nerve (Morita, Ito and Masai 1988). Following complete transection of the nerve, degeneration was observed in one root terminating in the anterior part of the ipsilateral glossopharyngeal lobe. The other root was found to arborise within the posterior pole of the lobe.

The tracer study reported here has shown afferent terminals in the anterior part of the glossopharyngeal lobe. The lobe would therefore appear to be topographically organised with respect to its afferent field.

In addition, the present study provides prima facie evidence for the bilateral innervation of the hyoid protrusion. However, due to the position and structure of the protuberance, the definitive unilateral delivery of the tracer was not possible. The presence of labelled afferents bilaterally in the medulla may, therefore, have been the result of bilateral spread of the tracer.

The observation of degeneration solely within the ipsilateral glossopharyngeal lobe following unilateral rhizotomy of the glossopharyngeal nerve (Morita et al 1988) would support this possibility.

The musculature of the hyoid structure has been

shown to be innervated by motor neurons originating within the glossopharyngeal motor nucleus and the anterior division of the vagal motor nucleus.

These motor nuclei exist as a continuous longitudinal column of cells in the medulla and exhibit no clear intermediate boundary (Ariens Kappers, Huber and Crosby 1936). In view of this continuity, and the distribution of cells within both the glossopharyngeal and anterior vagal divisions, any functional subdivision of the labelled cells would be arbitrary.

The involvement of both glossopharyngeal and vagal components in the efferent and afferent supply to the hyoid region does not coincide with the prediction made in the introduction on the basis of previous limited data. For methodological reasons, it may not, however, indicate that the prediction was wrong (assuming of course that the original interpretation of the data was sound). It has already been noted that the presence of label bilaterally in the medulla following injections of WGA-HRP into the hyoid protrusion is probably due to spread of the tracer across the midline of the structure. Spread of the tracer into surrounding regions on the floor of the pharynx may also explain the labelling of both vagal and glossopharyngeal components. Taste buds in these neighbouring zones in Ameirus melas are innervated by branchiomeric branches of the vagus (Landacre 1907).

The structure of the hyoid protrusion appears to be basically similar to that of the palatal organ. That is, they both consist of an epithelial layer containing a population of taste buds, and a subepithelial layer of intrinsic musculature.

The position of the two structures on opposing faces of the oropharyngeal cavity is suggestive of complimentary functional roles in feeding behaviour.

The hyoid protrusion is found in a position opposing the more rostral zone of the palatal organ and the central connections of this palatal zone are found in the anterior region of the vagal lobe.

Thus, the close relationship between the neurons innervating the hyoid protrusion and those innervating the anterior palatal organ may reflect the apparent association during feeding. In a recently published report of the central relationships of neurons innervating the gills, which oppose the lateral extents of the palatal organ, similar observations of a close relationship between neurons innervating opposing pharyngeal loci have been indicated (Morita and Finger 1985).

In addition, the present study provides further evidence for the topographical representation of the pharyngeal tissues within the medulla oblongata of cyprinoids, anterior pharyngeal locations being represented in the glossopharyngeal/anterior vagal lobe. This

coincides with the data of Morita and Finger (1985) showing a topographic representation of the gill arches along the same axis.

From a methodological standpoint, at least two alternatives exist to explain the variation in labelling observed between animals in the present study.

Firstly, if considered in relation to the post-injection survival periods, the results may be construed as being due to the presence of two phases of orthograde and retrograde transport (Table 7).

Table 7

Animal	survival period	reaction product detected
PL17	4 days	orthograde & retrograde
PL33	8 "	retrograde
PL34	8 "	retrograde
PL21	9 "	none
PL22	9 "	low density ortho- & retrograde
PL23	12 "	densest ortho- & retrograde
PL24	12 "	" " "
PL26	11 "	" " "

A primary phase, and the more rapid, might explain the occurrence of orthograde and retrograde label

following a four day survival. The disappearance and reappearance of both forms of label throughout the 8 - 9 day period may reflect the degradation of the rapidly transported tracer and the subsequent arrival of more slowly transported tracer at the terminals.

This slow phase could be responsible for the increase in density of the reaction product following the eleven and twelve day survival periods through the gradual accumulation of the transported tracer.

This variation with survival time was not observed in the experiments involving tracer delivery into the barbels or palatal organ. However, such lengthy periods were not generally used in those experiments. Also, the physical distance required for transport from the barbels or palate to the hindbrain may not have been sufficient to allow the detection of a fractionation of different rates of transport. Previous reports of the rates of axonal transport have described fast and slow rates in the orthograde direction but generally assume a single rate of retrograde transport in mammals (Grafstein & Forman 1980). Perhaps lower vertebrates possess an additional phase of transport.

Nevertheless, despite the possibility of equating the data to a two-phase sequence of transport, this approach assumes consistent delivery of tracer. The delivery procedure however was problematic. It is possible therefore, that the variation observed between animals

was related to the quantity of tracer available for transport.

In summary, these preliminary investigations of the innervation of the hyoid protrusion have provided limited data, probably because the delivery of the tracer was not consistently accomplished.

However, pooling the data from all animals, the primary afferent neurons innervating the hyoid protrusion terminate at a location in the medulla oblongata which is at the border of the glossopharyngeal and vagal lobes. The striated muscle fibres in the hyoid protrusion are supplied by motor neurons situated in the glossopharyngeal and anterior vagal motor nuclei. These observations suggest that the anterior regions of the ventral surface of the pharynx are represented at a level in the medulla which corresponds with that of the opposing pharyngeal surface, the anterior palatal organ. Such an organisation might not be surprising in view of the association of the two surfaces during feeding.

CHAPTER 6

THE ORGANISATION OF THE PALATAL AFFERENT INNERVATION

INTRODUCTION

In the previous sections, transganglionic transport of both WGA-HRP and HRP has been demonstrated in teleost cranial nerves following delivery into the peripheral site of innervation. It has been shown that the WGA-HRP conjugate is taken up and transported in greater detectable quantities in the peripheral-to-central direction than is the unconjugated enzyme.

The palatal organ is innervated by the palatine branches of the vagus nerve. In this palato-vagal system of the carp the transport of tracer in the peripheral-to-central direction has been used to demonstrate the central relationships in the vagal lobes of sensory afferents supplying receptors of the palatal organ (Chapter 3).

The purpose of the experiments reported here is to ask whether these tracers are also transported transganglionically in the reverse direction i.e. from the vagal lobes to their peripheral endings in the palatal organ. Transport in the central-to-peripheral direction has been demonstrated for unconjugated HRP in mammals and has been utilised successfully for tracing peripheral nerve endings

in the trigeminal system of rats (Marfurt & Turner 1983). However, WGA-HRP has not previously been used for a similar purpose in any vertebrate. If the same mechanisms of uptake and transport exist for the central-to-peripheral direction, the added efficiency of the conjugate should allow superior detection of peripheral nerve fibres and their distribution among receptors.

Although staining of normal tissue may answer some of the questions regarding the receptor innervation at the light microscopic level, the use of transported tracer can be used to specifically label the elements of relevance. Thus the second aim of these studies is to selectively demonstrate the peripheral distribution of sensory nerve endings supplying the palatal organ of the carp. In addition, this might be expected to show the distribution of the structures which the innervation supplies.

The palatal organ has been described as "being crowded over its entire extent with taste buds" (Herrick 1904; also see initial Introduction). Other studies of the distribution of teleost taste buds have only described where they occur and/or their density in those particular regions (e.g. Landacre 1907; Herrick 1901; Atema 1971; Kiyohara, Yamashita and Kitoh 1980; Ezeagor 1982; Marui, Evans, Zielinski and Hara 1983). No observations on their arrangement in those loci have been presented. The present studies of the afferent distribution may therefore, by association, provide information concerning taste bud

arrangement in the palatal organ.

Finally, the central projections of the primary afferents supplying the palatal organ have been demonstrated previously to be organised in a reticular fashion across the vagal lobes (Chapter 3). Those studies also showed that palatal coordinates were topographically represented in a columnar fashion through the layers of the vagal lobes. Thus, the third aim of this study is to ask if the peripheral distribution of palatal afferent endings gives an indication of the basis for the organisation of the central palatal representation.

MATERIALS AND METHODS

For this study of the palatal afferents, tracer was injected into the vagal lobes of nine animals.

The animals were initially anaesthetised in a solution of ethyl m-amino benzoate (1:5000 in water) until opercular movements ceased. They were then weighed and measured for length (snout to caudal peduncle).

The tissue overlying the dorsal surface of the cranium was removed by scraping with a clean scalpel blade. The area of cranial bone above the region of the medulla oblongata was then removed using a dental drill fitted with a saw attachment. This bone fragment was retained in teleost saline.

The fish was loosely wrapped in damp tissue paper and secured in a surgical apparatus. It was artificially respired by superfusing the gills with water containing anaesthetic at a similar concentration to that used for the initial anaesthesia. Once secured, the adipose tissue present over the hindbrain was aspirated so as to provide direct access to the dorsal surface of the vagal lobes.

A detailed account of the procedure for tracer delivery into the vagal lobes is provided in Chapter 7, describing the central nervous connections of this structure. The only difference in the procedure required for the present purpose was the delivery of larger volumes of the tracer.

Briefly, a tracer-filled Hamilton microsyringe (5 µL) was clamped in a micromanipulator and positioned above the surface of the vagal lobe to be injected. The plunger of the syringe was depressed until tracer was visible at the tip of the needle. This excess was gently removed. The syringe needle was then advanced to the surface of the vagal lobe and then a further distance of between 0.5 and 1.0mm. into the tissue. A volume of tracer between 40 and 50 nL. was ejected, and the syringe was maintained in position for approximately 5 minutes so as to minimise tracer reflux along the needle track.

This operation was performed four or five times along the complete rostro-caudal axis of the vagal lobe in order to deliver tracer throughout its entire length. The total volume of tracer thus injected into the vagal lobe was 200 nL. in all cases. This relatively large total volume (compared with that delivered for tracing the central connections of the vagal lobe) was regarded as necessary so as to optimise the opportunity for detecting transganglionically-labelled palatal afferents.

In five animals (CV11, CV12, CV23, CV24, CV25), WGA-HRP (1% or 2% in teleost saline) was injected into one vagal lobe only.

In the remaining four animals (CV19, CV20, CV21, CV22), HRP (50% in 1% lysolecithin) was additionally injected into the contralateral vagal lobe. Lysolecithin (L-lysophosphatidylcholine - Sigma) was included because

some evidence suggests that the presence of this detergent aids the uptake or transport of HRP (Frank, Harris and Kennedy 1988). The same injection procedures were followed for HRP delivery.

In this manner, the subsequent transganglionic transport of the individual tracers could be compared within the same animal. Also, the results of the delivery of one tracer served as a control for the results of delivery of the other.

On completion of tracer delivery, the cranial cavity was loosely packed with gelatin sponge (Sterispon) saturated with teleost saline. The cranial bone fragment was replaced and fixed in position using cyanoacrylate glue and dental cement.

The fish were revived with running tap water and maintained post-operatively in aquaria at a temperature of 18-20 °C. The survival periods ranged from three to seven days with the longer survival times resulting in the densest labelling. Table 8 indicates the experimental details for this group of animals.

Table 8

Animal number	Weight (gm)	Length (cm)	Tracer	Volume (nL)	Survival (days)
CV11	101	15.0	1% WGA-HRP	200	4
CV12	98	14.8	1% WGA-HRP	200	4
CV19	132	16.6	Left lobe		
			1% WGA-HRP	200	
			Right lobe		7
			50% HRP	200	
CV20	131	16.5	As CV19	200	7
CV21	152	17.2	As CV19	200	6
CV22	154	18.0	As CV19	200	3
CV23	106	15.2	1% WGA-HRP	200	7
CV24	98	15.2	2% WGA-HRP	200	7
CV25	93	14.8	2% WGA-HRP	200	7

In the experiments involving injections of 50% HRP, this tracer was dissolved in 1% lysolecithin.

The procedure for perfusion fixation of the animals was as described previously (Chapter 2). Subsequent to perfusion, the palatal organ was dissected free from the roof of the oropharyngeal cavity and placed in fresh fixative for a further two hours.

In two experiments (CV24, CV25), the palatal organ was reacted in its intact condition so as to reveal directly the gross organisation of the palatal innervation. For this purpose, the time periods of the stages of histochemical reaction required for tracer detection were extended. The pre-incubation soak was extended to 40 minutes and the incubation was allowed to continue for 30 minutes. The gross organisation of reaction product in the intact palatal organ was viewed and photographed through an Olympus operating microscope fitted with an Olympus photomicrographic system.

In some cases the palatal organ was embedded in 20% gelatin for sectioning in the transverse plane. On those occasions where the tissue was to be cut horizontally, embedding was not required. Sections were cut at 40, 60, 80 or 100µm. The histochemical detection of tracer was carried out on this sectioned material as previously described for CNS tissue (Chapter 2). No differences were apparent between embedded and unembedded tissues. All subsequent procedures were performed as previously described.

RESULTS

The palatal organ of the carp contains numerous taste buds within its epithelial layer which covers a network of intrinsic striated muscle fibrils. The CNS connections of the neurons innervating the individual elements within the palatal organ have already been shown to exist in the vagal lobes (Chapter 3).

In the present studies, injections of tracer into the vagal lobes resulted in HRP reaction product in the palatal organ. However, differences were apparent between the sites and density of reaction product following delivery of WGA-HRP or unconjugated HRP.

Injections of WGA-HRP into the vagal lobes resulted in labelled palatal sites in every experiment. In those experiments in which this tracer was injected into one vagal lobe only (CV11, CV12, CV23, CV24, CV25), reaction product was detected solely in the ipsilateral portion of the palatal organ. A vagal lobe therefore appears to be the central representation of the ipsilateral half of the palatal organ.

At the macroscopic level, reaction product in the intact palatal organ was observed in dense accumulations. The accumulations were found to be organised in clusters of varying numbers (Fig.6.1). The lowest number observed

was two densities forming an individual reactive site, whereas up to twenty densities were recorded within an apparently distinct cluster. Unlabelled regions were clearly visible between these reactive clusters. Thus the label was not homogeneously distributed across the palatal organ but confined to defined sites.

In some cases, a fortuitous lighting angle for illumination of the whole organ showed that the labelled sites appeared to correspond with a protrusion of the palatal surface beyond the adjacent unlabelled regions (Fig.6.2), presumably due to elevated papillae.

The precise arrangement of the clusters of reaction product was found to vary between medial and lateral palatal regions. In more medial palatal sites the reaction product was distributed in an apparently "spotted" arrangement of labelled clusters. Each cluster was surrounded by an unlabelled region (Fig.6.3 and Fig.6.4).

Far lateral palatal regions, however, were characterised by a continuity of labelled sites. The labelled clusters were not punctate as found in medial regions but formed a continuous network over the surface (Fig.6.5 and Fig.6.6). Thus, in contrast to the medial region, where labelled clusters were surrounded by a connected network of unlabelled regions, in the lateral palatal margin the labelled clusters encompassed unlabelled "cores". The distribution of label in the more medial regions of the palatal organ therefore appears to be reversed with

Figure 6.1

This photomacrograph shows the arrangement of labelled sites in the medial palatal organ following injection of WGA-HRP into a vagal lobe and subsequent histochemical processing of the whole palatal organ. The labelled sites in this medial palatal zone are organised in clusters of varying numbers.
Scale bar: 300µm.

Figure 6.2

This photomacrograph of a lateral region of the palatal organ shows that labelled sites are correspondent with a protrusion of the overlying epithelium.
Scale bar: 200µm.

Figure 6.1

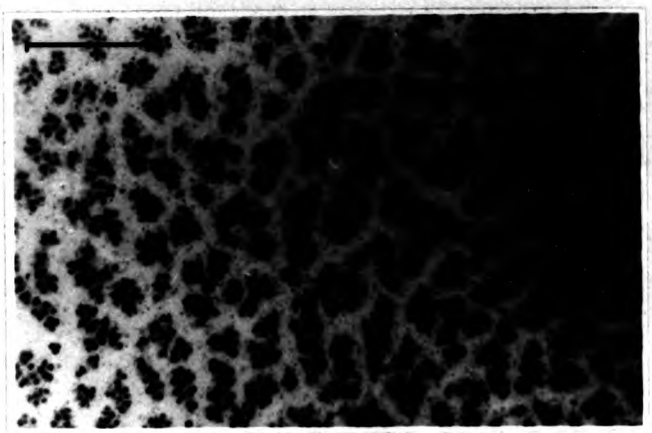


Figure 6.2

Figure 6.3

The clustered nature of the reaction product can be seen in this photomacrograph of a medial palatal site.
Scale bar: 160µm.

Figure 6.4

This higher power photomacrograph of a medial palatal site shows more clearly the individual labelled densities which constitute each cluster.
Scale bar: 100µm.

Figure 6.3

Figure 6.4

Figure 6.5

In this photomacrograph the "continuous" (rather than clustered) nature of the labelled densities can be seen in a lateral palatal site (L). In contrast, the label in a more medial location (M) exhibits clustering.
The tissue has received small incisions in order to allow complete infiltration of the histochemical reagents.
Scale bar: 100µm.

Figure 6.6.

In this higher power photomacrograph of a lateral palatal location the label can be clearly seen in a mesh-like arrangement.
Scale bar: 100µm.

Figure 6.5

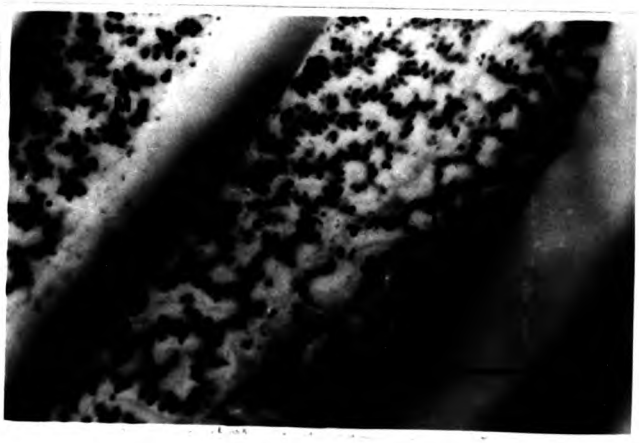


Figure 6.6

respect to the pattern in the lateral region.

In thick horizontal and transverse sections (60, 80 and 100μm.), clusters of reaction product were clearly visible (Fig.6.7 and Fig.6.8). In counter-stained tissue, they were observed to be at the level of the basal lamina separating epithelial from underlying tissues (Fig.6.9).

In some sections, labelled regions appearing to protrude beyond the level of the immediately adjacent palatal surface was observed (Fig.6.8). This may reflect the presence of elevated papillae at these locations but it could also be a fixation or embedding artefact.

In addition to dense reactive clusters, a much feinter form of reaction product was observed at a level subjacent to the dense accumulations (Fig.6.8). It was also observed between the clusters at this level (Figs.6.10 and 6.7). This form of the reaction product was distributed in a linear manner suggestive of accumulation within fibres.

No label was detected within the network of striated muscle fibrils forming the "body" of the palatal organ despite the previous description of a motor innervation to this network from the vagal lobes (Chapter 3).

Thus, the reaction product observed, both dense and feint, appears to represent the arrangement of the primary afferent supply to the palatal organ. In view of the orientation of the feint label in a fibre-like manner

Figure 6.7

A horisontal section (i.e. parallel to the palatal surface) showing the clusters of labelled densities from a medial palatal site. In addition to these densities, label suggestive of accummulation within fibres can be seen within and between clusters (F).
Scale bar: 100µm.

Figure 6.8

A transverse section through some labelled clusters in a medial palatal site. Note that the epithelium over the labelled regions protrudes beyond that between labelled clusters. Also, reaction product likely to represent labelled primary afferents (F) is present in association with the dense clusters.
Scale bar: 100µm.

Figure 6.7

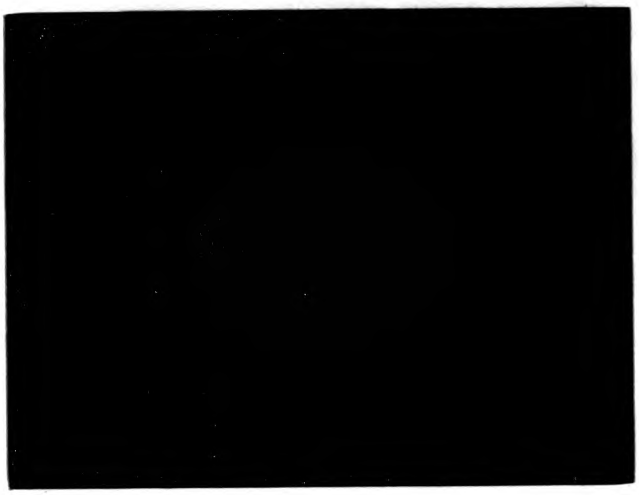


Figure 6.8

Figure 6.9

A transverse section showing the labelled densities. The boundary between the epithelium and the underlying tissue is indicated by the broken line.
A feinter, more diffuse form of the reaction product can be seen below the dense profiles.
Scale bar: 20μm.

Figure 6.10

A horizontal section showing the feinter form of reaction product associated with the densities and in addition between clusters.
Scale bar: 100μm.

Figure 6.9

Figure 6.10

coursing between clusters, its position adjacent to the epithelial layer and its association with the dense accumulations within the epithelial layers, this form of the label may indicate the course of the primary afferent neurons.

Similarly, the points of dense label would seem to identify the sites of termination of the primary afferent neurons. The degree of label observed at these points suggests the presence of dense plexuses of endings in these positions.

The requirement of thick sections in order to optimise detection of the reaction product prevented the description of the precise details of the pattern of fibre distribution to each labelled cluster. That is, it was not possible in the available material to determine whether the individual labelled components of a cluster were the terminations of single fibres or whether a single fibre branched to terminate as more than one component of a cluster.

In the experiments in which WGA-HRP was injected into one vagal lobe and unconjugated HRP was injected into the contralateral lobe (CV19, CV20, CV21, CV22), the superior efficiency of the conjugate for transganglionic transport was evident.

As already described, reaction product was consistently observed in those palatal regions ipsilateral to the site of WGA-HRP delivery. In contrast, the density

of the reaction product in the palatal sites ipsilateral to the HRP-injected vagal lobe was much more variable between animals. It was never as dense as that observed following WGA-HRP delivery. This occurred despite a fifty-fold greater concentration of the unconjugated HRP.

Also, the variation in reaction product density following HRP delivery was evident within an individual palatal organ; some palatal regions appeared more densely labelled than others. The reason for this is unclear. It would be surprising to find that afferents terminating in different regions of the vagal lobe had varying levels of efficiency for internalising HRP. It may be more likely that the variation reflected a heterogeneous exposure of the vagal lobe tissue to the tracer.

Despite the differences in density of reaction product following delivery of the individual tracers, the same sites were generally detected in both cases. However, in the palatal regions labelled with transported WGA-HRP, an additional epithelial locus of reaction product was observed in a position immediately superficial to the sites of dense accumulations (Fig.6.11). This additional label was never detected in palatal regions following HRP delivery into the corresponding vagal lobe.

These "fingers" of reaction product were only detected in association with the clusters of dense accumulations and were oriented loosely orthogonally to the palatal surface. Due to the thickness of section

Figure 6.11

A transverse section through the region of a labelled cluster showing not only the labelled densities and the subjacent labelled afferents (F), but also fine "fingers" of label (*) apparently associated with taste buds. Scale bar: 25μm.

Figure 6.11

required in order to observe this reaction product, it was not possible to unambiguously define the nature of the structure(s) with which it was associated. However, the arrangement of the reaction product was some times reminiscent of the gross morphology of a taste bud.

On a number of occasions, this reaction product did not appear to be continuous with the subjacent dense accumulations. An intervening region devoid of label appeared to be present. In other instances however, this unlabelled region did not appear to be present; the labelled sites seemed to be continuous with each other.

In summary, injections of WGA-HRP or HRP into the vagal lobes resulted in transport of the tracer to the ipsilateral region of the palatal organ.

The clustered distribution of reaction product in medial palatal sites was different to that observed in far lateral sites. In medial sites the numbers of dense accumulations of reaction product constituting a cluster varied. Dense accumulations were not clustered in lateral palatal sites but organised in a continuous network.

Finally, in palatal regions labelled with transported WGA-HRP, superficial "fingers" of reaction product were detected in association with the dense accumulations. These were not observed following HRP injections into the vagal lobes.

DISCUSSION

The palatal organ of the carp has previously been shown to be innervated by neurons projecting to the vagal lobe. This was demonstrated employing the transport of WGA-HRP to the CNS following delivery into the palatal organ (Chapter 3).

The study reported here has shown that both WGA-HRP and unconjugated HRP can also be successfully transported transganglionically in the reverse direction i.e. from central regions to the peripheral site of innervation.

Unconjugated HRP has previously been shown to be useful for defining the peripheral pattern of connections (Marfurt & Turner 1983). However, this seems to be the first example of the use of WGA-HRP for transganglionic tracing of primary afferent neurons to their peripheral origin. Its utility for elucidating the organisation of the peripheral innervation has been confirmed.

The observation of reaction product solely in the ipsilateral regions of the palatal organ following tracer injection into a vagal lobe indicates that the neurons innervating the palatal organ are connected solely with the ipsilateral vagal lobe. This confirms the result obtained from the experiments employing tracer injection into the palatal organ (Chapter 3).

The predominant form of reaction product observed was aggregated as a dense accumulation at the level of the

basal lamina. Electron microscopical studies of the carp taste bud (Uga & Hama 1967) and the barbel taste bud of Ictalurus (Reutter 1978) have identified a nerve fibre plexus at this position. The dense accumulations of reaction product therefore appear to represent labelled nerve plexuses. These plexuses are the terminal structures of the taste bud nerve and, in the barbel of Ictalurus, each individual plexus is associated with a single taste bud (Reutter 1978).

Each dense accumulation of reaction product in the palatal organ is therefore, by association, likely to represent the site of a taste bud in the overlying epithelium. On this basis, the present study has shown that the distribution of taste buds in the carp palatal organ can be determined using the transganglionic transport of WGA-HRP from the site of afferent termination in the CNS. It has also been shown that this distribution differs according to palatal sector.

In medial palatal sites, taste bud distribution appears to be clustered whilst in more lateral sites the organisation appears to be of a reticular form. No other studies of the precise distribution of taste buds in the palatal epithelium seem to be available. Other studies have described the gross distribution of taste buds in terms of density (Herrick 1901; Landacre 1907; Atema 1971; Kiyohara, Yamashita and Kitoh 1980; Ezeasor 1982; Marui, Evans, Zielinski and Hara 1983) but details of their

spatial relationships with each other were not presented. Thus, the receptor organisation, as assessed here in terms of the distribution of the peripheral innervation, remains to be confirmed using other techniques which stain the taste buds independently.

Nevertheless it appears that, in the palatal organ of the carp, taste buds are not homogeneously distributed throughout the epithelium but confined to defined locations. The number of taste buds in these sites varies considerably from one palatal position to another.

The epithelial region associated with the dense reaction product has been shown, in both the intact organ and transverse sections, to be capable of protruding beyond the general level of the adjacent unlabelled tissue. It is possible that this represents the presence of papillae which are elevated above the level of the surrounding epithelium.

In addition, it has previously been shown that the palatal organ has the ability to selectively protrude regions of its surface in response to the presence of a stimulus (McGlone 1977; Edgar 1983; personal observation). The present observations may therefore alternatively indicate that clusters of taste buds can themselves protrude independently beyond the neighbouring surface. It might be expected that these distortions of the palatal surface would present a larger receptive area for sensory evaluation of material in the oropharynx.

The functional consequences of the variation in spatial relationships between the taste buds, however, remains obscure. As a working hypothesis it might be postulated that the variation is related to different functional requirements during the feeding process and the different occlusal surfaces encountered in the oropharyngeal cavity by medial and lateral palatal regions.

The medial portions of the palatal organ oppose the floor of the cavity. Excluding the region of the small hyoid protrusion, this ventral surface is a bony, ridged structure. The lateral palatal margin opposes the region of the gill arches and rakers.

As regards their respective roles in feeding behaviour, the medial and lateral palatal sites appear to differ. Much of the grinding and crushing of material involved in mastication occurs between the palatal organ and the pharyngeal floor. This operation appears to be minimal between the lateral surface of the organ and the gill arches (Sibbing 1982).

The relatively high clustered density of receptors in the medial region of the organ may reflect the greater opportunities for sensory evaluation of material in this region during the masticatory process. In contrast, the oropharyngeal region between the gill arches and the palatal organ appears to function as a component of a branchial sieve. In this region, material is filtered by the gill arches and large particles are retained prior to

being swallowed (Sibbing 1982).

Thus, within the feeding process, the medial and lateral palatal regions seem to be involved in different functions. The variation in receptor arrangement may reflect these different functional roles.

The course of the neurons terminating as the dense plexuses subjacent to taste buds has also been visualised in the present studies. A single branch of the nerve innervating the palatal organ appears to supply more than one cluster. However, the precise details of the pattern of the palatal innervation to individual taste buds were not clear from the relatively thick sections. That is, questions regarding the branching of the neurons, whether to individual plexuses within a cluster or to different clusters of plexuses were not answerable from the present material. In mammals (Miller 1971) and the frog (Rapuzzi & Casella 1965), single nerve fibres have been shown to respond to stimulation of more than one taste bud. Morphological studies have also shown that single gustatory neurons receive input from more than one taste bud in mammals (Miller 1974). Whether the pattern of taste bud innervation in the carp palatal organ is comparable may be assertained from semi-thin and thin sections of labelled palatal regions.

Comparison of the density of reaction product following WGA-HRP injections into the vagal lobe with that following the injection of equivalent volumes of unconjug-

ated HRP into the contralateral lobe, has again shown that the conjugate is more efficiently taken up and transported transganglionically (cf. Chapters 3 & 4). Although labelling of peripheral sites was detected following HRP delivery, the reaction product associated with WGA-HRP delivery was much denser despite a fifty-fold lower concentration of the latter tracer. Thus, the superiority of the conjugate in tracing from a peripheral-to-central direction is also apparent in the reverse direction.

A comparison of the loci of reaction product detected following delivery of the individual tracers has shown a difference between them at the superficial epithelial level.

Injections of WGA-HRP into the vagal lobe resulted in reaction product in cellular components associated with the subjacent nerve plexus. This was not found in experiments employing HRP injections. Although the density of label following HRP injections was generally low, it was present in sufficient amounts to expect the observation of all the labelled sites detected in the experiments employing WGA-HRP.

The reason for this difference in sites of reaction product associated with the individual tracers may be related to taste bud structure and synaptic organisation.

The teleost taste bud possesses various cell types. Firstly, elongated cells arranged orthogonally to the epithelial surface extend from the basal part to the apex

of the taste bud (Reutter 1978). These cells have been subdivided into sustentacular and receptor cells, the former being defined as supporting cells. However, it has been suggested that "compelling evidence for such a functional distinction does not yet exist" (Reutter 1982). Nevertheless, ultrastructural studies of the taste buds from a number of teleost species do indicate a variety of different types based on morphology and cytoplasmic inclusions (Reutter 1982; Toyoshima, Nada and Shimamura 1984). The functional consequences of these structural differences remain undetermined.

The remaining cell type, the basal cell, is found at the base of the taste bud and is oriented along an axis parallel to the basal lamina i.e. perpendicular to the receptor and sustentacular cells. The nerve fibre plexus extends between the basal cells and the processes of the receptor cells (Reutter 1982).

The reaction product observed in the epithelial layer corresponding to the positions of taste buds has been shown to be oriented orthogonally to the palatal surface. This observation would seem to rule out the basal cells of the taste bud as the structures in which the tracer has been sequestered.

Alternatively, the orientation of the reaction product could reflect its presence in component cells of the taste bud similarly oriented i.e. receptor or sustentacular cells. It could also represent the position

of labelled primary afferent neurons ramifying amongst these taste bud components.

However, if the latter were true, these same features would be expected to be labelled following the injection of unconjugated HRP into the vagal lobes. This was not the case and it would therefore appear that the labelled site does not correspond to primary afferent arborisation within the taste bud. (This, of course, assumes that the volume of tracer transported in those experiments employing HRP would have been adequate to detect these features).

The remaining candidate for the site of the reaction product is the receptor/sustentacular cell type. The question which arises is why should this cell type be labelled following WGA-HRP delivery and devoid of label subsequent to HRP delivery?

The WGA-HRP conjugate has been shown on a number of occasions in mammalian sensory systems to be capable of being transported trans-synaptically. The tracer is transported across the synaptic cleft to the postsynaptic neuron (Itaya and Van Hoesen 1982). In contrast, unconjugated HRP has rarely been shown to possess the same capacity. The presence of reaction product within component structures of the taste bud following WGA-HRP injections but not HRP injections may be due to this differential capability.

This might however be expected to predict the

presence of efferent synapses onto the relevant taste bud component. Efferent synapses onto sensory cells have been reported within the barbel taste bud of Ictalurus (Desgranges 1966: in Reutter 1982), but other observations of efferent synapses in teleost taste buds have not been reported.

If efferent synapses onto the carp palatal taste bud were present, tracer delivery into the palatal organ and subsequent transport to the ipsilateral vagal lobe may be expected to identify their origin. Such experiments have previously shown the presence of labelled soma (Chapter 3; Morita and Finger 1985). Although these soma have been interpretted as the origins of the innervation of the palatal musculature, it might be initially postulated that a proportion of them represent an efferent supply to palatal taste buds.

However, in the present experiments, if the tracer had been taken up and transported by these putative efferent neurons, it would have been expected to be similarly taken up by the innervation of the palatal musculature. No reaction product was observed associated with the striated muscle fibrils.

In view of this lack of label in the motor innervation and the paucity of studies reporting efferent synapses at the teleost taste bud, it may be wise to dismiss the possibility that label in the taste bud has been transported across an efferent synapse. However, what

alternative explanation is available?

A body of evidence exists which suggests that innervation is a requirement for the maintained integrity of the mammalian taste bud. Nerve transection (Donoso & Zapata 1976) and reinnervation experiments (Oakley 1974) have shown that the sensory nerve is responsible for this maintenance. Efferent fibres of the gustatory nerve are not required (Berland, Chu, Hosley, Jones, Kaliszewski, Lawler and Oakley 1977). In addition, blockage of axonal transport in the sensory nerve results in the degeneration of taste buds (Sloan, Hughes and Oakley 1983).

Thus, it appears that the mammalian taste bud shows a neurotrophic dependency upon its sensory innervation. It has been suggested that a neurotrophic agent may be transported along gustatory sensory axons and subsequently released at axon terminals (Sloan et al 1983).

If the palatal taste buds of the carp were similarly dependent upon the sensory innervation for the maintenance of their structural integrity, a similar neurotrophic effect may exist. Barbel taste buds in catfish have been shown to be dependent upon their sensory innervation for maintaining their morphological integrity (Geraudie & Singer 1977). A trophic substance might be released from the sensory neuron and diffuse to its effector site(s) thereby sustaining the taste bud.

The reaction product in the receptor/sustentacular cell type could therefore result from the transport of

WGA-HRP from the sensory afferents in this manner.

The observation of reaction product within the taste bud structure following WGA-HRP injections into the vagal lobe and its absence following HRP injections may simply be due to the far greater efficiency of the processes responsible for the internalisation of the former into the primary afferent neurons. There would therefore be a greater quantity available for "trans-cellular" transport once the tracer had been transported to the distal ends of the neurons.

If this transcellular diffusion of the tracer does indicate the presence of an equivalent process for neuro-trophic maintenance of the taste bud, it may eventually be useful in isolating and characterising the neurotrophic substance.

Finally, one of the aims of the present studies was to ask if the distribution of palatal afferents indicated an organisation upon which the central representation of the palatal organ is based.

The palatal organ of the carp has been shown to be topographically mapped onto the vagal lobes. In addition, this map has been found to exist throughout the depth of the vagal lobes. Palatal coordinates are therefore represented in radial columns of the vagal lobe neuropil (Chapter 3).

The terminals of the primary afferent neurons

supplying the lateral and intermediate palatal organ have previously shown to be organised in a reticular fashion in the superficial sensory layers of the vagal lobe (Chapter 3: Fig.3.10). In view of the topographical representation, it might be argued that each component unit of this network represents a distinct palatal region. The projection of this unit through the layers of the vagal lobe may therefore represent an individual "functional column".

The organisation of endings in the lateral palatal organ bears a strong resemblance to the reticular organisation of afferent terminals in the topographically-appropriate regions of the vagal lobe (Chapter 3: Figs.3.8 and 3.9). Intuitively, this correspondence in the arrangement of the peripheral and central terminals suggests the presence of a representation in the vagal lobe which replicates the distribution of the palatal receptors.

As regards the medial palatal innervation, the present study has demonstrated that afferent endings are not distributed homogeneously throughout the epithelium but arranged in clusters of varying numbers.

If the reticular organisation of primary afferent endings in the vagal lobes does reflect the actual distribution of receptors at the palatal surface, this would suggest that the organisation must be modified in order to similarly represent the receptors at medial

palatal locations. Although this does not necessarily indicate the absence of a reticular organisation in regions representing medial palatal sites, it might suggest that the dimensions of the reticular components are much reduced and thereby reflect the clustered arrangement of receptors in medial palatal sites. This interpretation gains support from the observation that reaction product in the superficial layers of the vagal lobe observed following injections of WGA-HRP into medial palatal sites is much more densely accumulated than that following similar injections into lateral palatal sites (compare Figs.3.15A and 3.15B). Thus, it may be the case that the varying distribution of afferent endings in the palatal organ is reflected in the organisation of their central terminations.

It would be interesting to design further tracer studies in order to specifically compare the fine details of the organisation of the central projections of afferents supplying the medial and lateral palatal sites so as to clarify this issue.

In summary, the experiments reported here have demonstrated that the peripheral distribution of cranial nerve afferents can be successfully visualised following injections of WGA-HRP or HRP into the region of their central terminals. The former tracer has been shown to be superior for this purpose.

Components of the taste bud structure have been shown to sequester WGA-HRP, but not unconjugated HRP, following injections into the ipsilateral vagal lobe. This has been related to the possible transcellular diffusion, from afferent endings, of a trophic substance required for the maintenance of the structural integrity of taste buds.

The gross distribution of afferents in the palatal organ has been shown to vary according to palatal region. The relationship between the peripheral distribution of palatal afferent terminals and the organisation of their map in the vagal lobes suggests that the organisation of the central terminals may be a replication of the taste bud distribution on the palatal organ.

CHAPTER 7

CENTRAL CONNECTIONS OF THE VAGAL AND FACIAL LOBES

INTRODUCTION

In previous chapters the vagal, facial and glossopharyngeal lobes of the carp hindbrain have been shown to receive primary afferent input from accumulations of taste buds in distinct "gustatory regions". Afferents from the barbels have been shown to project to the ipsilateral facial lobe whereas those innervating oropharyngeal structures terminate in the vagal or glossopharyngeal lobes. The internal and external gustatory systems of the carp have therefore been shown to be mapped somatotopically onto their sites of primary projection. Likewise, the facial and vagal lobes of the Ictalurus catfish have been shown to receive external and internal gustatory input respectively (Morita and Finger 1985). The primary gustatory inputs to the medulla therefore appear to be generally segregated according to the site of gustatory receptors.

Classical studies of the gustatory projections in Cyprinus carpio have described the central efferents from and afferents to the medullary lobes in normal tissue only (Herrick 1905; Barnard 1936). These studies have indicated

the presence of two efferent projections each from the vagal and facial lobes. A descending secondary projection to upper spinal levels was described in addition to an ascending secondary gustatory projection to the superior secondary gustatory nucleus. This isthmic nucleus is highly developed in species with equally well-developed external and internal gustatory systems. That is, species such as the cyprinoids and siluroids which possess dense accumulations of taste buds on barbels and in the oropharyngeal cavity.

Studies of normal tissue are not capable of providing definitive evidence of any topographical organisation within central nervous projections. However, ascending efferents from the facial lobe of Cyprinus carpio have been described previously to course in the medial portion of the ascending secondary gustatory tract whilst vagal lobe efferents are found laterally in the tract (Barnard 1936).

In view of the different behavioural roles apparently subserved by the different groups of gustatory afferents to the individual medullary lobes of Cyprinus carpio, it might not be unexpected to find that their somatotopic representation in the hindbrain is maintained at higher levels in the central nervous system.

This organisation has been observed in Crucian carp (Morita, Ito and Masai 1988; Morita, Murakami and Ito 1983) and in the Bullhead catfish (Finger 1978; Morita & Finger

1985).

Normal studies have also described the presence of descending afferents to the vagal and facial lobes from the diencephalic inferior lobes in Cyprinus carpio. However, due to the intricate intermingling of fibres and the relative diffuseness of the projection, precise details of the diencephalic nuclei involved were not available. In Ictalurus (Finger 1978) and Crucian carp (Morita et al 1988; Morita et al 1983), these nuclei have been identified and observed to consist of both "vagal" and "facial" portions. Thus, the diencephalic nuclei with projections to the medullary lobes also appear to be topographically organised. In these species, therefore, the higher central connections of the vagal and facial lobes appear to be entirely organised in a topographical manner.

In view of these previous observations, a similar topographical organisation of the central efferents and afferents of the vagal and facial lobes in Cyprinus carpio might be expected. The purpose of the experiments reported here is therefore to identify these central connections and to test the hypothesis that the somatotopical projections of the primary afferent inputs onto the hindbrain demonstrated in the previous chapters is maintained within the central gustatory pathways of Cyprinus carpio.

The classical study of Herrick (1905) identified the course of the axons from the diencephalon in the tractus lobo-bulbaris. At the level of the vagal lobes this

tract was observed to ramify in close association with the motor nuclei. However, the precise level at which they terminated was not discussed.

Delivery of HRP into the vagal lobes exposes various layers to the tracer solution. A precise determination of the level of the vagal lobes in which the descending afferents terminate is therefore precluded in such experiments.

In Cyprinus carpio. retrograde transport studies have shown that cells in the region of the nucleus lobobulbaris project to the trigeminal and facial motor nuclei (Luiten and Van der Pers 1977). The design of these studies did not provide details of the vagal lobe projections from this region. Thus, the medullary organisation of the descending efferents from the diencephalon of Cyprinus carpio is not known.

In order to accomplish this, a procedure for the localisation of the source of descending diencephalic efferents is required. Regarding this requirement, an electrophysiological method for the localisation of diencephalic sites with projections to the ipsilateral vagal lobe has previously been developed (Beach and Roberts; personal communication).

Briefly, during antidromic electrical stimulation of the descending input to the vagal lobe, ipsilateral diencephalic structures have been localised by recording through a tungsten electrode. The response of the relevant

cells to this form of stimulation has been characterised.

A procedure for localisation of diencephalic regions which project to the ipsilateral vagal lobe therefore appears to be available. By using a tracer-filled micropipette as the recording electrode for localisation, the tracer may, theoretically, be accurately delivered by subsequent ionophoretic expulsion from the micropipette.

An additional purpose of the experiments reported here is therefore to use this electrophysiological approach to trace the descending projection from the diencephalon to the ipsilateral vagal lobe.

MATERIALS AND METHODS

These investigations of the central connections of the vagal and facial lobes involved 22 carp. Similar procedures were adopted for tracer delivery into both the facial and vagal lobes. They are therefore dealt with simultaneously in this section.

All animals were initially anaesthetised in a solution of ethyl m-amino benzoate in water (at a dilution of approximately 1:5000) until respiratory movements ceased. Measurements of weight and length (snout to caudal peduncle) were taken.

The tissue overlying the dorsal cranium was removed by scraping with a clean scalpel blade. The area of the cranium above the hindbrain was removed using a dental drill fitted with a saw attachment and this bone fragment was retained in teleost saline.

After wrapping the animal loosely with damp tissue paper it was clamped securely around the pectoral region in a surgical apparatus to ensure minimal movement during the operative procedures. The animal was artificially respired by superfusing the gills with anaesthetic solution at a similar dilution to that employed for the initial anaesthesia. In order that an adequate flow of this superfusate could be maintained, it was important to ensure that free movement of the opercular flaps was not hindered.

The adipose tissue present over the hindbrain was

aspirated so as to provide direct access to the relevant region of the medulla oblongata. It was necessary to aspirate carefully because, as occurred on several occasions, damage to the semicircular canals could be inflicted during this procedure.

Injections of HRP solution were made via a 5 µL Hamilton syringe fitted with a 33-gauge needle and performed under an Olympus binocular microscope. Having filled the syringe to the 1 µL level, it was secured in a micromanipulator and directed over the relevant zone of a vagal or facial lobe. The control necessary for expulsion of precise nanolitre (nL) volumes of HRP solution was not adequately provided by manual depression of the syringe's plunger. A second, hydraulically-driven manipulator equipped with a Perspex extension rod was therefore positioned above the former to give the necessary fine regulation of the plunger's movement (Fig.7.1).

Immediately prior to advancing the needle into the required anatomical site, a small volume of fluid was expelled from the syringe in order to ensure that the system for tracer delivery was operational. When this fluid had been cautiously removed, the tip of the needle was positioned precisely over the facial or vagal lobe and advanced to the surface.

For facial lobe injections, the needle was directed to a position intermediate between the midline and the lateral border of the lobe (Fig.7.2 and Fig.7.3). This

Figure 7.1

This diagram illustrates the procedure for making injections of HRP into a facial or vagal lobe. The micro-syringe is held in manipulator A and depression of the plunger is controlled by a perspex rod held in manipulator B which is advanced by means of a hydraulic microdrive. In this way, very small volumes of tracer can be expelled from the syringe consistently and simply.

Figure 7.1

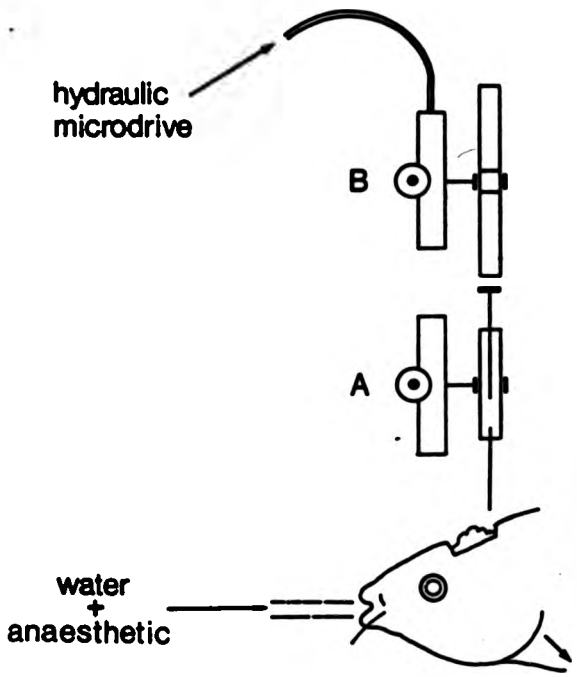


Figure 7.2

This illustration of a sagittal section through the carp brain shows the method of tracer delivery for facial and vagal lobe injections.

Figure 7.2

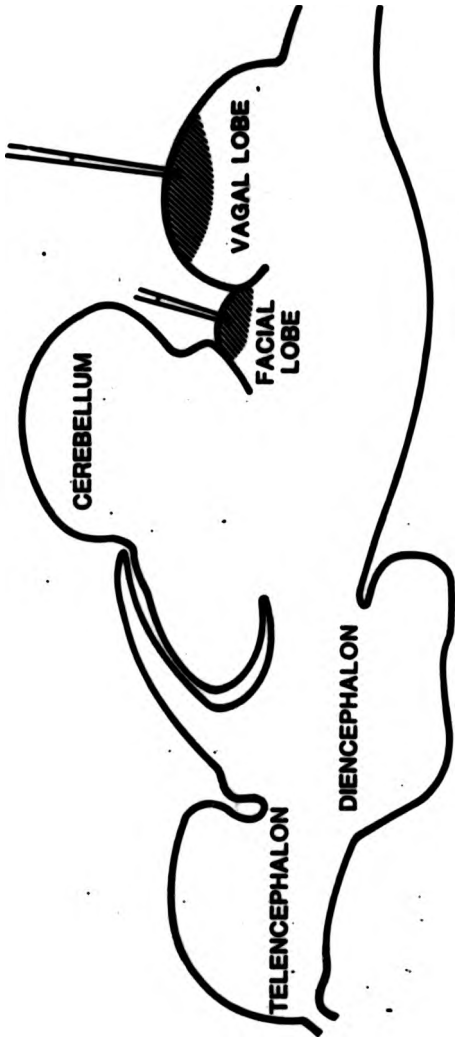
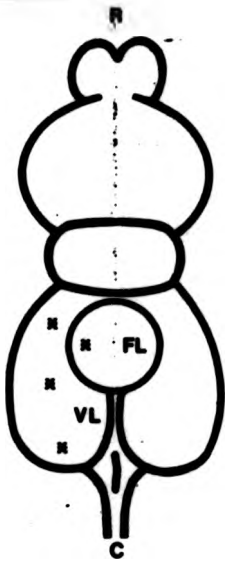

VAGAL LOBE
FACIAL LOBE
CEREBELLUM
DIENCEPHALON
TELENCEPHALON

Figure 7.3

This diagram shows a dorsal view of the carp brain and indicates the position of tracer injections into the facial lobe (FL) or vagal lobe (VL).
R: rostral. C: caudal.

Figure 7.3



approach was taken in order to minimise the likelihood of the tracer spreading across the midline or into the ipsilateral glosso- pharyngeal/anterior vagal lobe neuropil. For vagal lobe injections, the needle was directed to a range of positions along the rostro-caudal axis of the lobe. Injections into the rostral limit of the vagal lobe included the ipsilateral glossopharyngeal lobe.

After penetrating the surface, the needle was advanced into the neuropil. HRP solution (20-30%) was then expelled from the syringe. The depth of the tissue at which tracer was delivered varied between 0.25 and 1.5mm, but was normally at 0.5mm.

To avoid excessive reflux along the track of the advancing needle and on to the surface of the brain, the tracer was delivered in aliquots of 25 nL, with a period of approximately 5 minutes between each delivery. After the final delivery, the needle was left <u>in situ</u> for between ten and thirty minutes. Total volumes delivered ranged from 25 nL. to 100 nL.

After carefully retracting the needle, the surface of the brain adjacent to the injection site was gently swabbed with a piece of dry gelatin sponge (Sterispon) or tissue paper. This procedure was employed in order to "mop up" any tracer which had spread on to the surface of the brain during delivery.

The intracranial cavity was then loosely filled

with gelatin sponge saturated in teleost saline. The cranial bone was replaced and secured with cyanoacrylate glue and dental cement.

Postoperatively, the animals were revived with fresh water and maintained in aquaria at 16-22°C for three or five days prior to sacrifice. All subsequent procedures for detection of transported tracer were as described in Chapter 2.

The individual experimental features for each animal are shown in Table 9 (facial lobe injections) and Table 18 (vagal lobe injections).

TABLE 9 (Facial lobe injections).

Animal number	length (cm)	weight (gm)	volume (nL)	tracer	survival (days)
CF01	13.5	78.4	100	20% HRP	3
CF02	14.3	85.1	100	20% HRP	3
CF03	13.7	77.7	100	20% HRP	3
CF04	13.5	78.5	100	20% HRP	3
CF05	15.0	99.3	50	20% HRP	3
CF06	14.7	81.9	50	20% HRP	3
CF07	13.4	73.1	50	30% HRP	3
CF08	14.0	84.8	50	30% HRP	3
CF09	15.3	99.8	50	30% HRP	3
CF10	15.5	99.9	50	30% HRP	3
CF11	14.2	92.0	25	25% HRP	5
CF12	14.0	84.0	25	25% HRP	5

TABLE 10 (Vagal lobe injections).

Animal number	length (cm)	weight (gm)	volume (nL)	tracer	survival (days)
CV01	13.6	77.0	100	20% HRP	3
CV02	14.3	102.0	25	25% HRP	5
CV03	14.1	103.0	25	25% HRP	5
CV04	14.8	94.0	25	25% HRP	5
CV05	13.7	85.0	25	25% HRP	5
CV06	14.8	96.0	50	25% HRP	3
CV07	14.0	94.0	100	25% HRP	3
CV08	14.8	116.0	50	30% HRP	3
CV09	14.4	110.0	100	30% HRP	3
CV10	17.2	122.0	75	30% HRP	3

The experiments on the descending projections from the diencephalon involved 21 animals. The preparatory surgical procedures were precisely as described for delivering tracer into the facial and vagal lobes. The experimental arrangement for the localisation of the diencephalic sites projecting to the hindbrain, and tracer delivery is illustrated in Figure 7.4. As can be seen from this diagram, three separate "modules" constituted the procedures:

1. Electrical stimulation of the vagal lobe.
2. Recording of the response in the diencephalic tissue.
3. Tracer delivery into the localised zone.

Electrical stimulation:

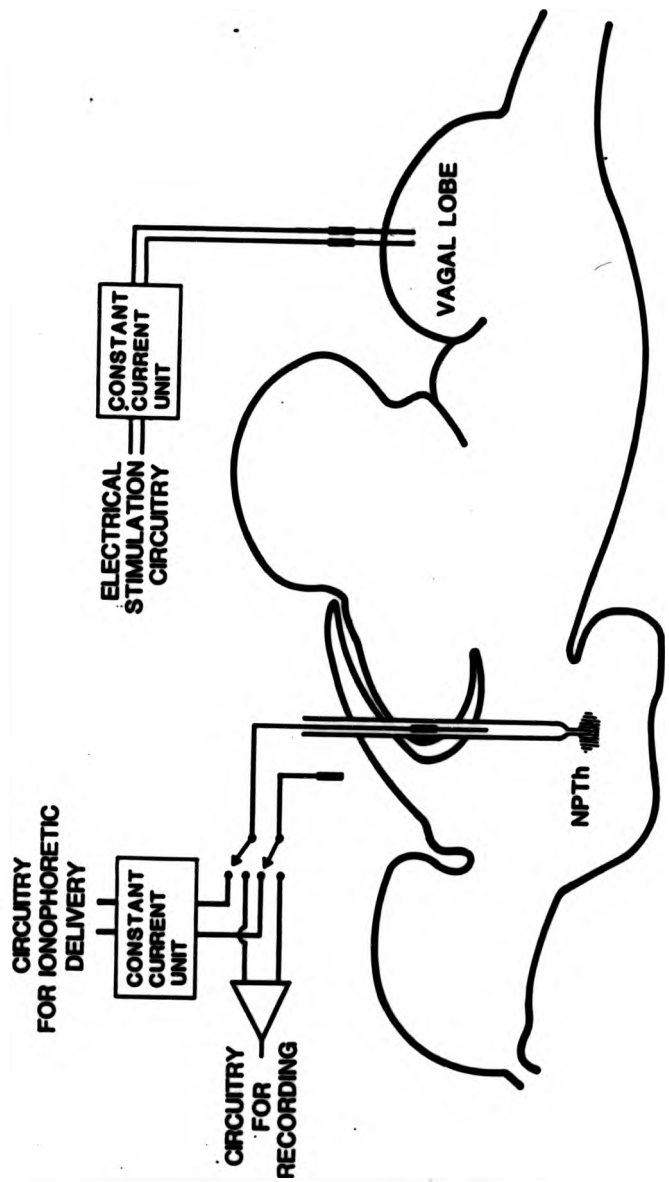
Bipolar tungsten electrodes held in a micromanipulator were advanced into the vagal lobe to a depth of between 0.5mm. and 1.0mm.

The parameters of the electrical stimulation pulses were generated by a suitable arrangement of electronic modular units (Neurolog). These devices were arranged so as to regulate the frequency of stimulation (NL303 - Period generator) and the period of stimulation (NL403 - Pulse width). The output of these modules was fed to the bipolar stimulating electrodes through a Pulse buffer (NL510) and Stimulus isolator (NL800) which determined current level. The stimulating current was 100 µA. and was applied for 2 msec. This was repeated at a frequency of 1 Hertz during

Figure 7.4

This illustration of a sagittal section through the carp brain shows the experimental arrangement for 1) electrical stimulation of the vagal lobe, 2) recording of the evoked responses in the region of the nucleus posterior thalami (NPTh), and 3) ionophoretic expulsion of the tracer. For other details see text.

Figure 7.4


CIRCUITRY FOR IONOPHORETIC DELIVERY
CONSTANT CURRENT UNIT
CIRCUITRY FOR RECORDING
ELECTRICAL STIMULATION CIRCUITRY
CONSTANT CURRENT UNIT
VAGAL LOBE
NPTh

recording.

Recording:

Potentials were recorded through micropipettes pulled from 1.2 mm. borosilicate glass (Kwikfill). Fine tipped pipettes were pulled using a Mecanex BB-CH horizontal puller. The tips were carefully broken to produce an internal diameter of between 10 µm. and 25 µm.

Micropipettes were filled with 20% HRP or 1% WGA-HRP (in teleost saline or 0.9% NaCl). They were held in a Neurolog electrode chamber (NL101) filled with the same electrolyte as utilised for the tracer solution. Signals from the microelectrode were fed via a unity gain Neurolog headstage amplifier (NL100) into an AC preamplifier (NL104) and then an AC-DC amplifier (NL106). The indifferent silver chloride electrode was inserted into exposed cervical tissue.

Signals were filtered from 100 Hz. to 10 KHz., displayed on a Tektronix 5113N D13 storage oscilloscope or Gould OS4040 digital oscilloscope which was triggered by the output of NL303 used for regulation of stimulation frequency. The signals were stored on FM tape using a Racal instrumentation tape recorder (Store 4DS) at 15 inches sec, DC-10K band width. For the purposes of obtaining a hard copy of the relevant electrophysiological data, the output of the tape recorder was fed into a Bryans X-Y plotter (26000 A4). Averaged data was provided by feeding stored

data from the tape recorder into a Neurolog Averager (NL750).

The microelectrode was advanced slowly into the diencephalic tissue using a hydraulically-driven manipulator fitted with a micrometer screw gauge for depth discrimination. The tip of the electrode was regarded as having penetrated a diencephalic site with projection to the vagal lobe when a characteristic signal "locked" to the stimulus was recorded. The optimum signal was considered to be the evoked waveform with the greatest amplitude. This optimum response was recorded at a depth between 2.9 mm. and 3.9 mm. from the surface of the optic tectum.

Tracer delivery:

The connection of the tracer-filled recording electrode was switched to the positive pole of a Neurolog Stimulus isolator (NL800) which provided constant current conditions (Fig.7.4). The indifferent electrode was switched to the negative pole of the device via a series digital multimeter (Keithley 169). This arrangement allowed direct evaluation of the ionophoretic current level.

The circuitry used for generating the ionophoretic current was the same as required for the stimulation of the vagal lobe. Only the parameters of the current pulse altered.

The maximum ionophoretic current used was 3 µA. The current was applied every 10 seconds for a period of five

seconds i.e. the "ON" period was 5 seconds and the "OFF" period was 5 seconds. The total "ON" time ranged from 5 to 7.5 minutes. In a large proportion of the experiments it was not found possible to maintain a current of 3 μA. throughout the "ON" time. This was assumed to be due to blockage of the pipette tip. In those cases where the current level dropped below 1 μA. the current was reversed for a period of approximately 30 seconds whereupon the maximum 3 μA ionophoretic current could usually be applied again. This operation generally unblocked the tip. However, in the cases where this operation was necessary, it was usually required several times during the experiment.

On completion of the ionophoretic delivery of the tracer, the micropipette was maintained in position for approximately 10 minutes before being carefully retracted.

In addition to this electrophysiological approach to HRP delivery, pressure injections were made in several animals using a Hamilton microsyringe (5 μL) fitted with a 33 gauge needle. The delivery technique was as described for injections of tracer into the facial and vagal lobes. For the present purposes however, the needle was directed into the region of the diencephalic region with projections to the hindbrain. A stereotaxic procedure was not utilised due to the lack of an atlas of the carp diencephalon. Instead, the needle was positioned using the experience gained from the ionophoretic approach. A volume of 50 nL.

of the tracer (20% HRP or 1% WGA-HRP in teleost saline) was expelled at a depth of 3.2 mm. from the surface of the optic tectum. The needle was maintained in position for 10 minutes prior to retraction.

After tracer delivery, the intracranial cavity was losely filled with gelatin sponge (Sterispon) saturated with teleost saline. The cranial bone fragment was replaced and secured with cyanoacrylate glue and dental cement. The fish were revived with fresh tap water and returned to aquaria maintained at 18.5 - 21 °C for the postoperative survival period. All subsequent procedures were as described in Chapter 2.

The experimental details for each animal receiving tracer in diencephalic sites are listed in Table 11. Unfortunately, due to processing difficulties, no photographs of the hindbrain label obtained following diencephalic tracer delivery were available.

TABLE 11

Animal number	Weight (gm)	Length (cm)	Tracer	Delivery	Survival (days)
NP1	119	16.0	20% HRP	"Iono"	5
NP2	111	16.2	" "	"	3
NP3	110	16.0	" "	"	3
NP4	124	16.0	1% WGA-HRP	"	3
NP5	113	15.9	" "	"	3
NP6	112	15.7	" "	"	3
NP7	111	16.3	" "	"	3
NP8	113	15.5	" "	"	3
NP9	170	18.8	" "	"	5
NP10	156	17.5	" "	"	2
NP11	123	16.0	" "	"	4
NP12	124	15.8	" "	"	4
NP13	101	15.0	20% HRP	Pressure	4
NP14	108	16.0	1% WGA-HRP	"	4
NP15	98	15.4	" "	"Iono"	4
NP16	96	14.8	20% HRP	"	3
NP17	93	15.2	1% WGA-HRP	"	7
NP18	122	16.2	" "	Pressure	6
NP19	130	16.2	20% HRP	"Iono"	4
NP20	127	16.8	" "	"	2
NP21	145	17.0	1% WGA-HRP	"	4

RESULTS

Connections of the facial lobe

Injections of HRP solution into the facial lobe resulted in reaction product indicative of both the orthogradely- and retrogradely-transported tracer.

Orthogradely-labelled sites:

Following injections, labelled fibres were observed ventrolaterally of the ipsilateral half of the facial lobe. One group of these fibres was observed to turn caudally whilst the other group turned in the rostral direction (Fig.7.5). These two bundles constituted the descending and ascending secondary gustatory tracts as described by Herrick (1985). They were only observed ipsilateral to the site of injection. It was not possible to determine in these experiments whether the two bundles represented collaterals of the same neurons or separate projection neurons.

The descending secondary gustatory tract was observed to course in the medial portion of the descending spinal trigeminal tract. It terminated in the ipsilateral medial funicular nucleus.

The ascending secondary gustatory tract terminated at the level of the superior secondary gustatory nucleus. The terminal label was observed bilaterally in the medial one-third of the nucleus (Fig.7.6). In some cases labelled

Figure 7.5

A horizontal section through the medulla oblongata at a level ventral of the facial lobe. Fibres of the ascending (ASGT) and descending secondary gustatory tract (DSGT) are labelled.

C: caudal. R: rostral.

Scale bar: 150µm.

Figure 7.6

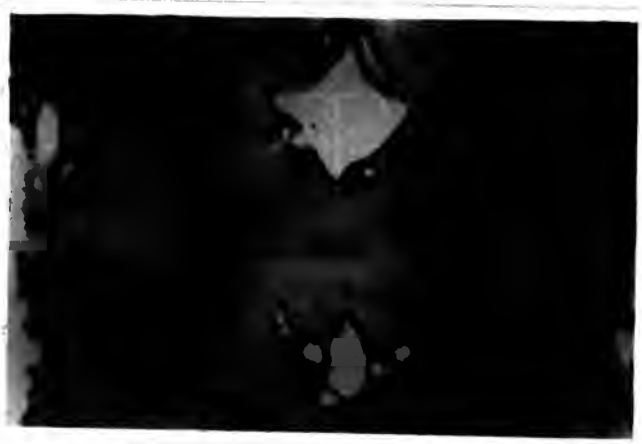
A horizontal section through the superior secondary gustatory nuclei and their commissure (SSGN) showing orthograde reaction product (O) bilaterally in the medial zone of the nucleus following injection of HRP into the facial lobe.

R: rostral. C: caudal

Scale bar: 250µm.

Figure 7.5

Figure 7.6



fibres were observed in the commissure of this nucleus.

Ascending afferents from the facial lobe were therefore observed to project in the ipsilateral ascending secondary gustatory tract and to terminate bilaterally in the superior secondary gustatory nucleus.

A group of fibres could also be observed diverging away from the ascending gustatory tract and appeared to be coursing rostrally in a position ventral to the superior secondary gustatory nucleus. This fibre bundle could not be traced further rostrally than this isthmic region. It was therefore unclear whether this bundle of fibres terminated in this region or represented retrogradely-filled axons of the labelled diencephalic soma (see below). No orthogradely-transported tracer was consistently detected in any position rostral of the superior secondary gustatory nucleus.

Retrogradely-labelled sites:

Retrogradely-labelled neuronal cell bodies were only observed in diencephalic nuclei. In general they were observed bilaterally but labelled cells contralateral to the site of injection were few in number, and faintly labelled.

Three cell groups were identified as being labelled:

1. In the dorsomedial inferior lobes, cells of the nucleus posterior thalami (Sheldon 1912) were labelled. Within this group of neurons, only those soma in the caudal fraction

contained retrogradely-transported HRP (Fig.7.7).

2. A more diffuse group of neurons situated ventro-laterally of the nucleus posterior thalami was labelled. This group, the nucleus lobo-bulbaris, was not labelled throughout its entire rostrocaudal extent. Only cells in the caudal region of the nucleus were found to be labelled (Fig.7.8). This nucleus was originally referred to as the nucleus cerebellaris hypothalami (Sheldon 1912) due to the belief that it received cerebellar efferents. However, it appears that this nucleus does not receive an input from cerebellar efferents (Finger 1978a) and it is generally referred to as the nucleus lobo-bulbaris (after Wallenberg 1907).

3. Small cells of the nucleus diffusus lobi lateralis (Sheldon 1912) were also retrogradely-labelled. In common with the position of labelled cells in the nucleus posterior thalami and nucleus lobo-bulbaris following facial lobe injections, only cells in the more caudal zone of the nucleus diffusus lobi lateralis were labelled.

Thus, the facial lobe receives descending afferents from three diencephalic nuclei, the nucleus posterior thalami, nucleus lobo-bulbaris and the nucleus diffusus lobi lateralis. Only the caudal fraction of each of these nuclei appears to provide afferents to the facial lobe.

Figure 7.7

A horizontal section through the nucleus posterior thalami (NPTh) ipsilateral to the site of facial lobe injection, showing the labelled cells only in the caudal zone of the nucleus. The remaining region of the NPTh is devoid of label.

NPgL: nucleus preglomerulosus pars lateralis.

C: caudal. R: rostral. M: medial. Scale bar: 200µm.

Figure 7.8

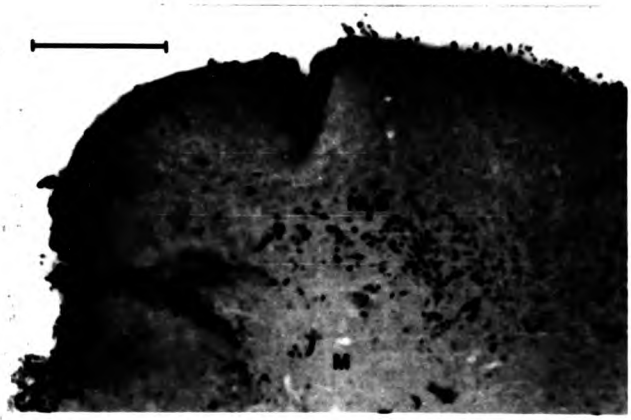
A horizontal section showing labelled cells in the caudal zone of the nucleus lobo-bulbaris (NLB).

C: caudal. R: rostral. M: medial.

Scale bar: 200µm.

Figure 7.7

Figure 7.8



Connections of the glossopharyngeal and vagal lobes

As observed following facial lobe injections, delivery of HRP into a vagal lobe resulted in reaction product indicative of both the orthogradely- and retrogradely-transported tracer (Fig.7.9).

Orthogradely-labelled sites:

Following injections of HRP, labelled axons were detected coursing ventrally of the vagal lobe in both descending and ascending directions.

The terminal site(s) of the descending fibres could not be definitively localised. However, as no retrogradely-labelled soma could be recognised at a caudal level it seems highly likely that the reaction product did represent descending efferents from the vagal lobe.

Ascending fibres terminated in the ipsilateral superior secondary gustatory nucleus. In contrast to the facial lobe projection to the nucleus, only the lateral two-thirds of the superior secondary gustatory nucleus was labelled following vagal lobe injections (Fig.7.10). The most lateral portion of the nucleus exhibited reaction product following injections into the most caudal region of the ipsilateral vagal lobe. This reaction product was observed to encroach more medially within the lateral "vagal" division of the superior secondary gustatory nucleus with increasingly rostral vagal lobe injections. No label was detected within the contralateral superior

Figure 7.9

A parasagittal section through the entire brain showing orthograde reaction product in the ascending secondary gustatory tract (ASGT) and the superior secondary gustatory nucleus (SSGN) ipsilateral to the site of injection. A labelled fibre bundle is present in a position ventrally of the superior secondary gustatory nucleus (arrowed). Retrograde label is also present in diencephalic sites (*) but is not recognisable as such at this magnification.
VL: vagal lobe. FL: facial lobe. Cb: cerebellum.
Tel: telencephalon. IL: inferior lobes.
Scale bar: 1mm.

Figure 7.18

A horizontal section through the superior secondary gustatory nuclei and their commissure (SSGN) showing orthograde label (O) present only in the lateral zone of the nucleus ipsilateral to the injected vagal lobe. Compare this figure with that showing the label in the SSGN following a facial lobe injection of HRP (Figure 7.6).
C: caudal. R: rostral.
Scale bar: 250µm.

Figure 7.9

Figure 7.10

secondary gustatory nucleus.

Thus, secondary gustatory neurons of the vagal lobes appear to project entirely ipsilaterally to the superior secondary gustatory nucleus and terminate in a distinctly different region of the nucleus to the facial lobe projection.

As observed following facial lobe injections, a small fibre bundle was observed coursing ventrally of the ipsilateral superior secondary gustatory nucleus. This bundle is visible in Figure 7.9. In the case of vagal lobe injections of the tracer, these fibres appeared to be directed dorsally of the inferior lobes. However, it was not possible to trace them any further than the isthmic region and no orthograde label was consistently detected rostrally of this position. Again, these fibres may have been retrogradely-filled axons from labelled diencephalic nuclei.

Retrogradely-labelled sites:

The same diencephalic nuclei were retrogradely labelled following vagal lobe injections as observed after injections into the facial lobe. That is, cells of the nucleus posterior thalami, nucleus lobo-bulbaris and nucleus diffusus lobi lateralis were retrogradely-labelled. However, distinctly different regions of the nuclei were labelled.

After vagal lobe injections, only the rostral zones of the nuclei were observed to exhibit retrogradely-

labelled soma.

In the nucleus posterior thalami, this "vagal" division of the nucleus was found to be larger than the "facial" division (Fig.7.11). Also, greater numbers of cells were labelled following vagal lobe injections.

In the nucleus lobo-bulbaris (Fig.7.12) and nucleus diffusus lobi lateralis the cells observed to exhibit reaction product were confined to the rostral zones of the nuclei.

Thus the vagal lobes of the carp receive descending afferents from the same diencephalic nuclei as the facial lobe. However, these medullary lobes receive their inputs from different regions of the nuclei. The diencephalic nuclei projecting to the facial and vagal lobes appear to possess vagal and facial subdivisions. Also, the descending afferents to the vagal lobe originate solely from the ipsilateral inferior lobes. In contrast, the facial lobe projection from the inferior lobes is mainly ipsilateral but appears to have a small contralateral component.

In view of the apparently sound theoretical basis for the electrophysiological studies and the availability of a promising technique for localisation of diencephalic sites with projections to the medulla, the quantity of anatomical data obtained using this approach was less than expected.

No single cause for the failure rate encountered

Figure 7.11

A horizontal section through the nucleus posterior thalami (NPTh) showing labelled cells occupy the rostral zone of the nucleus following HRP injection into the ipsilateral vagal lobe. The large densely labelled objects in the caudal zone of the nucleus are probably erythrocytes exhibiting endogenous peroxidase activity. Compare this figure with that showing the labelled cells in the NPTh following facial lobe injections (Figure 7.7).
NPgL: nucleus preglomerulosus pars lateralis.
C: caudal. R: rostral.
Scale bar: 200μm.

Figure 7.12

A horizontal section through the nucleus lobobulbaris ipsilateral to the injected vagal lobe. Compared with the results of HRP injection into the facial lobe (Figure 7.8), the labelled cells here occupy a more rostral position.
C: caudal. R: rostral.
Scale bar: 200μm.

Figure 7.11

Figure 7.12

was evident. The histochemical processing for transported tracer was carried out successfully in all cases, as adjudged by the reactivity of endogenous artefact in the tissue. This stage of the experimental procedure was therefore not viewed with suspicion.

The remaining features of the operation in which problems could have been encountered were: electrical stimulation of the ipsilateral vagal lobe, recording of the antidromically-elicited responses in the diencephalon, and the ionophoretic delivery of the tracer.

Electrical stimulation:

Evoked activity in the diencephalon was found impossible to record in only one experiment (NP9). Although this may have indicated a failure to antidromically stimulate the descending afferents to the vagal lobe, it may alternatively have been due to some feature of the recording of the evoked responses. The ionophoretic delivery of the tracer was accomplished in this experiment as gauged by the presence of a labelled injection site. However, no hindbrain label was detected.

In all the other experiments using the electrophysiological approach evoked activity was observed. It was therefore assumed that the electrical stimulation of descending afferents from the diencephalon had been successfully accomplished.

Recorded activity:

Although evoked responses were detected in all the

experiments but one, two features in particular were found to vary between experiments. Firstly, the number of identifiable components of the evoked waveform was not consistent from one experiment to another. Figure 7.13 and Figure 7.14. show the averaged optimum response for NP18 and NP15 respectively. It is clear from a comparison of these two traces that different numbers of components were recorded from these experiments. As the scale of the traces is identical, it is equally evident that the amplitude of responses also varied between experiments.

In addition, evoked responses were detected at widely varying depths in the diencephalic tissue. For example, the traces shown in Fig.7.13 and Fig.7.14 were obtained from recordings at depths of 3.25 mm. (NP15) and 3.8mm. (NP18).

It seems possible therefore, that the evoked waveforms recorded were not all derived from the descending afferents to the hindbrain.

Ionophoretic delivery:

Clearly identifiable ionophoretic delivery sites were detected in only five experiments (NP6, NP7, NP9, NP15, and NP16). Four of these utilised 1% WGA-HRP solution, whilst 20% HRP solution was ionophoresed in the remaining experiment.

Although evoked activity was detected using either tracer, the efficacy of the ionophoretic delivery procedure was greater using the conjugated tracer. This was probably

Figure 7.13.

This shows the averaged optimum response recorded from NP15 during antidromic stimulation of the descending projections to the vagal lobe.
It was recorded at a depth of 3.25 mm.

Figure 7.13

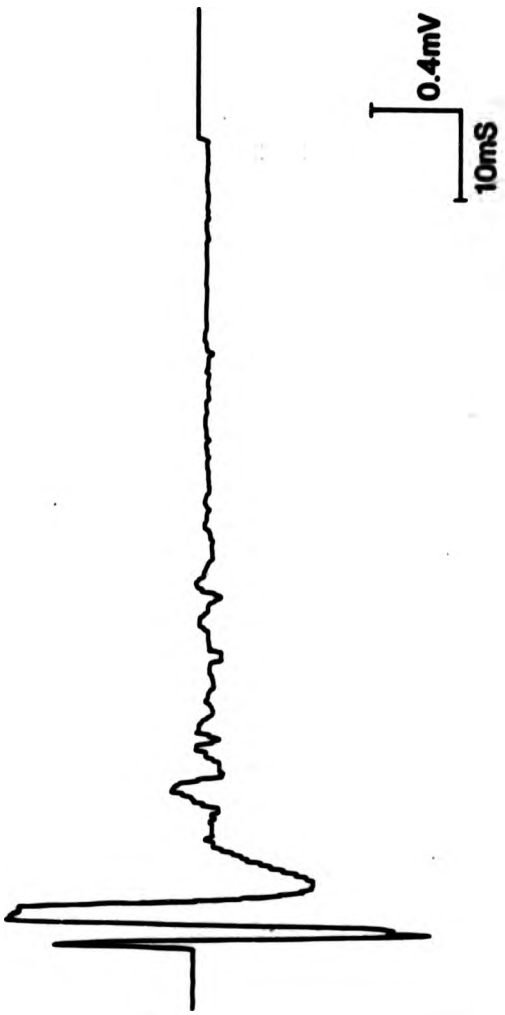
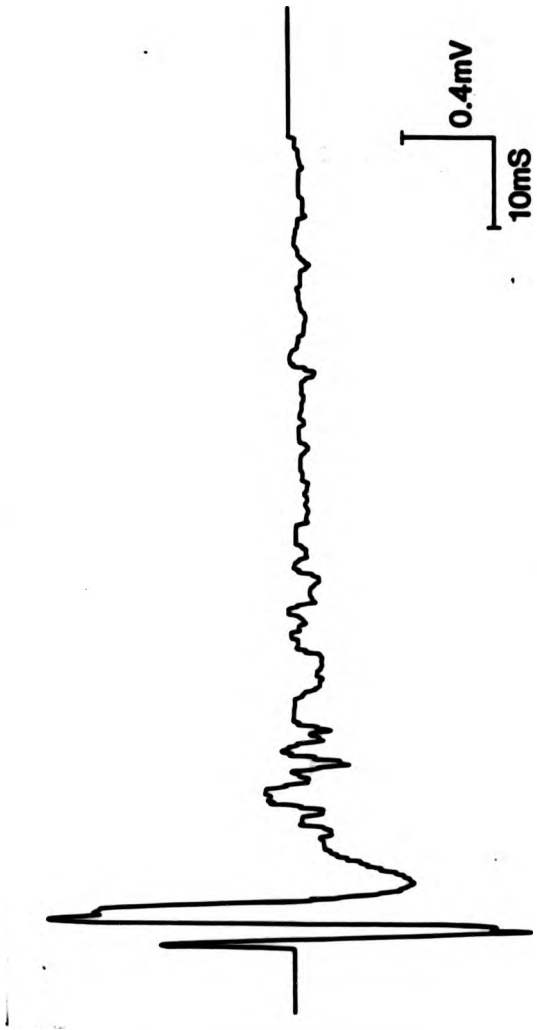


Figure 7.14

This trace shows the averaged optimum response recorded from NP18 during antidromic stimulation of the descending projections to the vagal lobe.
Comparing this trace with that shown for NP15 in Figure 7.13. it can be seen that these two optimum responses differ in the number of components and in the amplitude of those components.
Also. the best response available from NP18 was recorded at a depth of 3.8 mm.

Figure 7.14



due to the use of a lower concentration of the tracer in the case of the conjugate. Blockage of the tip was frequently encountered in experiments utilising unconjugated HRP. This presumably accounted for the lack of expulsion of adequate quantities of tracer in those experiments using this form of tracer. However, tip blockage was also encountered occasionally in experiments using WGA-HRP.

The issue of tip blockage therefore seems to explain the lack of detectable injection sites in all but five of the experiments. Nevertheless, it might be suggested that the ionophoretic current level and the time for which it was applied were below optimum. However, the observation that the values were adequate for five experiments would appear to negate this suggestion.

In two of the experiments in which an injection site was detected, transported tracer was also observed.

Following ionophoretic delivery of HRP into a diencephalic region where evoked activity was recorded, labelled fibres were observed coursing to the rostral limit of the ipsilateral vagal lobe (NP16).

The injection site was found to extend through the nucleus lobo- bulbaris and into the nucleus posterior thalami. The position of the labelled fibres was observed to coincide with the ventrolateral portion of the ascending secondary gustatory tract.

In view of the delivery of tracer into sites

previously shown to project to the facial and vagal lobes, the reason for the lack of detectable label in these structures was unclear. The fibres containing transported tracer could only be traced as far as the rostral extent of the ipsilateral vagal lobe.

Following ionophoretic delivery of WGA-HRP into a diencephalic region from which evoked responses were recorded, a distinct injection site was observed in the region of the nucleus lobo-bulbaris and nucleus posterior thalami (Fig.7.15). In addition, transported tracer was detected in the ipsilateral vagal lobe. This label was not found at all levels in the vagal lobe but confined to the deepest laminae of the sensory division (Fig.7.16). Label could not be traced clearly into the core of the facial lobe but terminated within its ventral neuropil. No label indicative of orthogradely-transported WGA-HRP was observed in the regions of the trigeminal or facial motor nuclei, as would have been predicted from the studies of Luiten and Van der Pers 1977. The projections to these motor nuclei may, however, be too diffuse to be detected by this method.

Figure 7.15

A horizontal section through the diencephalon of NP16 showing the injection site following ionophoresis of 1% WGA-HRP into a site identified electrophysiologically. The injection site includes the NPTh and NLB. Cells of the NPTh are heavily labelled following ionophoresis in this animal.

Scale bar: 200μm.

Figure 7.15

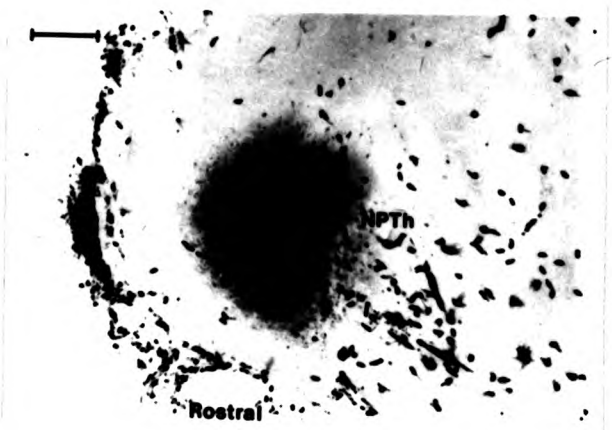


Figure 7.16

This series of diagrams from adjacent horizontal sections shows the distribution of orthograde label (shaded areas) in the vagal lobe ipsilateral to the site of ionophoresis in NP15 (Figure 7.14). The label was found in the deepest sensory laminae.

The upper left diagram shows the most ventral section in which label was observed.

Figure 7.16

DISCUSSION

The gustatory systems of the carp are greatly developed in comparison to other vertebrates. The dense populations of taste buds found on the external body surface and in the oropharyngeal cavity have been associated with the hypertrophy of the facial and vagal lobes respectively (Herrick 1905). In previous sections, this has been confirmed.

Specifically, afferent neurons supplying the individual barbels have been shown to project to relatively extensive regions of the ipsilateral facial lobe (Ch.4). Also, the innervation of the palatal organ on the dorsal surface of the oropharyngeal cavity has been shown to project topographically onto the ipsilateral vagal lobe (Ch.3).

Classical studies of the central connections of these medullary lobes were confined to observations based on normal material (Herrick 1905; Barnard 1936). The present studies have used a modern neuroanatomical tract tracing procedure to trace both the afferents and efferents of these structures.

The early studies defined the ascending output of the lobes as constituting the ascending secondary gustatory tract which terminates in the ipsilateral superior secondary gustatory nucleus. This has been confirmed in the present HRP studies.

In addition, it has been shown in these experiments that the superior secondary gustatory nucleus is topographically organised with regard to the inputs from the medullary lobes. The secondary neurons originating from the facial lobe terminate in the medial one-third of the nucleus. This projection has been shown to be bilateral, fibres crossing to the contralateral nucleus in the intranuclear commissure. In normal material, fibres of the ascending secondary gustatory tract have previously been observed coursing in the commissure to the opposite nucleus but their origin in the facial lobe was not clarified (Herrick 1905). The topographical arrangement of the superior secondary gustatory nucleus was suggested by Barnard (1936). His observations showed that the ascending gustatory tract from the facial lobe moves medially so that the majority of its fibres enter the nucleus in this medial position.

The remaining lateral two-thirds of the superior secondary gustatory nucleus has been shown in the present studies to receive input from the secondary neurons projecting from the glossopharyngeal and vagal lobes. These projections are entirely ipsilateral. More precisely, neurons projecting from the caudal zone of a vagal lobe terminate in the most lateral extent of the ipsilateral nucleus. Those originating in the glossopharyngeal and anterior vagal lobes project more medially in the ipsilateral nucleus. Thus, the medullary lobes are mapped

topographically onto the superior secondary gustatory nuclei. The somatotopical organisation of the primary afferent input to the medullary lobes therefore appears to be maintained within the secondary projections. Figure 7.17 provides a summary diagram including an illustration of the topographical organisation of these connections.

The superior secondary gustatory nucleus in other teleosts has been shown to be similarly somatotopically organised with respect to its input. The relative sizes of the "facial" and "vagal" components have, however, been shown to vary between species.

In the Crucian carp (Carassius carassius), the extent of the two inputs corresponds to that observed in the present studies for Cyprinus carpio (Morita, Ito and Masai 1988; Morita, Murakami and Ito 1983). In Ictalurus, however, the input from the vagal lobes is smaller in extent compared to afferents from the facial lobes. Tracer and degeneration studies have shown that the facial lobe input to the superior secondary gustatory nucleus of Ictalurus projects medially of the vagal input but this latter component is restricted much more laterally than is observed in cyprinoids (Finger 1978; Finger 1983).

Unlike the cyprinoids, Ictalurus possesses bilateral facial lobes. Their hypertrophy is associated

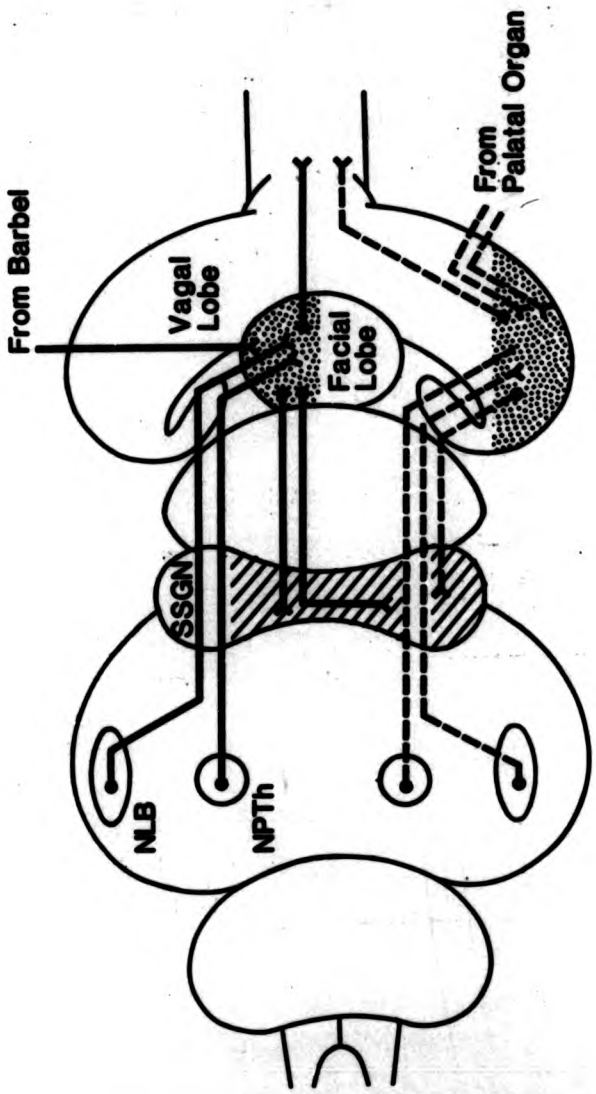
Figure 7.17

This summary horizontal section through the brain shows the inputs and outputs of the facial and vagal lobes and includes illustration of the topographical nature of the ascending projections to the superior secondary gustatory nuclei (SSGN).

NPTh: Nucleus posterior thalami.

NLB: Nucleus lobobulbaris.

Figure 7.17


From Barbel
Vagal Lobe
Facial Lobe
From Palatal Organ
SSGN
NLB
NPTh

with dense populations of taste buds on three bilateral pairs of barbels (Atema 1971). The external gustatory system of Ictalurus therefore appears to be more highly developed than its equivalent in cyprinoids.

In contrast, the hindbrain of the cyprinoid forms is dominated by the vagal lobes associated with the presence of the palatal organ. The vagal lobes of Ictalurus are much diminished in size relative to the situation observed in cyprinoids. This appears to correspond to the presence of a less highly developed internal gustatory projection in Ictalurus compared with the cyprinoids.

The differences observed between the secondary gustatory projections of the vagal and facial lobes in cyprinoids and Ictalurus would therefore appear to be representative of varying degrees of elaboration of the internal and external gustatory systems.

In the present studies, no orthogradely-transported HRP was detected in any position further rostral than the superior secondary gustatory nucleus. This nucleus therefore appears to be the anterior limit of the secondary projection from the vagal and facial lobes in the common carp. This also appears to be the situation in the Crucian carp (Morita et al 1988; Morita et al 1983).

In contrast, in Ictalurus a small bundle of the ascending secondary gustatory tract from the facial lobe continues rostrally to the ipsilateral diencephalon and

terminates in the nucleus posterior thalami and the vicinity of the nucleus lobo-bulbaris (Finger 1978; Finger 1985). Vagal lobe projections to these sites in Ictalurus are minimal (Finger 1985). Although the nucleus posterior thalami and nucleus lobo-bulbaris do not appear to be classically homologous with the similarly-termed nuclei of the common carp, it is evident that the common carp does not possess an ascending secondary gustatory projection to the diencephalon whereas such a projection is present predominantly within the facial lobe efferents of Ictalurus. It may be significant that the presence of the projection in Ictalurus coincides with a more highly developed facial system than found in the carp.

In the carp, diencephalic gustatory projections have been regarded as tertiary connections. The results of the current experiments are not contradictory to this suggestion. In studies of normal tissue, an ipsilateral projection from the superior secondary gustatory nucleus to the region of the lateral inferior lobes has been described (Herrick 1905; Barnard 1936). More recently, the terminations of this projection have been identified in the nucleus glomerulosus and nucleus diffusus lobi inferioris of the Crucian carp (Morita et al 1980; Morita et al 1983). Thus, the direct secondary projection to the diencephalon which exists in Ictalurus appears to be tertiary in the cyprinoids. Diencephalic gustatory projections in cyprinoids appear to be relayed from the medullary lobes

via the superior secondary gustatory nucleus.

The studies of retrogradely-transported HRP reported here have shown that three diencephalic nuclei project to the facial and vagal lobes of the common carp. These are the nucleus posterior thalami, the nucleus lobo-bulbaris and the nucleus diffusus lobi lateralis. The course of the desending axons from these nuclei could not be traced in the diencephalon. In normal tissue, the course of the descending efferents from the diencephalon have been described as constituting the tractus lobo-bulbaris. This tract was observed as passing ventrally of the superior secondary gustatory nucleus and merging into the ascending secondary gustatory tract (Herrick 1905). The present studies have shown a fibre bundle coursing in a position below the ipsilateral superior secondary gustatory nucleus. Although it could not be traced further rostrally, this labelled bundle may be the tractus lobo-bulbaris. In support of this, no orthograde label was observed rostrally of the superior secondary gustatory nucleus, thus appearing to eliminate the possibility that it represents ascending efferents from the medullary lobes. Also, no orthograde label was observed in the optic tectum, ruling out the presence of a lobotectal tract as described previously in some other species (Brickner 1930).

Caudally, the labelled bundle merges with the ascending secondary gustatory tract and cannot be

distinguished from the latter throughout its course from the hindbrain. Thus the bundle may represent the tractus lobo-bulbaris, but not enough evidence is available from these studies to categorically confirm this proposal.

The results of these experiments show that the diencephalic nuclei are organised topographically with respect to the hindbrain regions to which they project. Each nucleus has a "facial" and "vagal" component. In the case of all the nuclei, the more rostral portion projects to the vagal lobe whilst the caudal cells project to the facial lobe. It would be expected that the glossopharyngeal component would be found between these two. Figure 7.18 summarises these findings diagrammatically.

Descending afferents to the medullary lobes therefore appear to form distinct vagal and facial subgroups rather than collateral innervation. This organisation might not be unexpected due to the contrasting behavioural roles subserved by the different medullary lobes. The facial lobe appears to be an integrative centre for the localisation of nutrient material via taste buds on the external surface of the body (Herrick 1904; Atema 1971). The role of the vagal lobes in behaviour appears to be one of control of sifting and sorting prior to ingestion (Atema 1971).

This topographical organisation of the diencephalic nuclei which project to the medullary lobes of Cyprinus

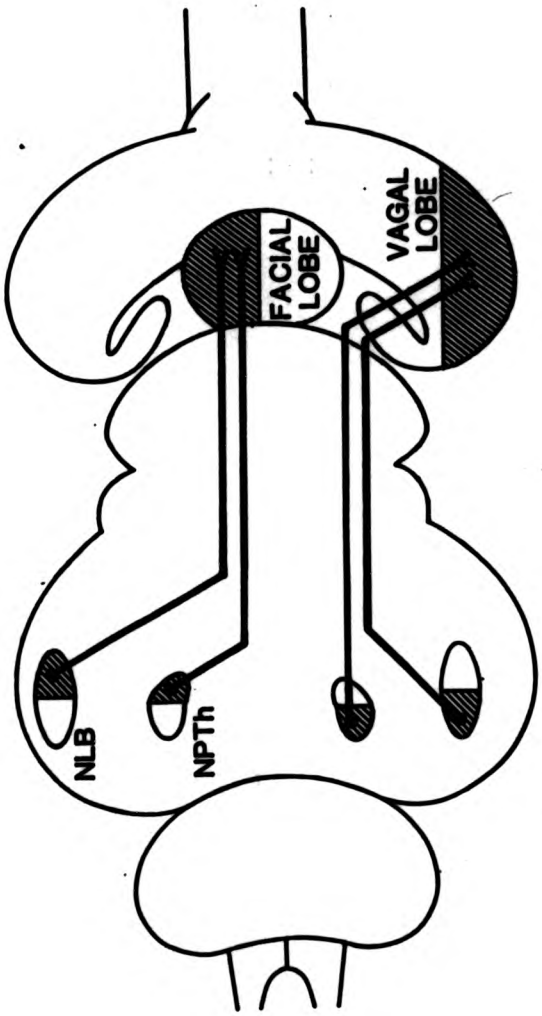
Figure 7.18

This diagram summarises in a schematic horizontal section the topographical organisation of the diencephalic projections to the facial and vagal lobes.

NPTh: Nucleus posterior thalami.

NLB: Nucleus lobobulbaris.

Figure 7.18



carpio has also been observed in other species. In the Crucian carp, the rostral versus caudal division observed in the current studies is also present (Morita et al 1983). In the bullhead catfish, the orientation of the topography is mediolaterally directed. The most lateral cells project to the ipsilateral vagal lobe whilst the medial group are labelled following injections of HRP into the facial lobe (Finger 1978; Morita & Finger 1985).

As observed for the ascending projections to the superior secondary gustatory nucleus, the relative sizes of the "vagal" and "facial" portions of the diencephalic nuclei varies between species. In the catfish, the diencephalic "facial" component is larger than the lateral "vagal" zone. In the carps, the cells projecting to the vagal lobes represent the largest fraction of the nuclei. This would, again, appear to be a reflection of the relative degrees of elaboration of the facial and vagal gustatory systems in the various species.

Thus although the general plan of the vagal and facial lobe connections in the different species is basically similar, various components are differentially developed as a consequence of the varying hypertrophy of the primary relays.

The results of the experiments carried out in order to trace the medullary terminations of the descending afferents from the diencephalon were disappointingly

limited. This appears to have been due to limitations of both the electrophysiological localisation method and the ionophoretic delivery procedure. If more time had been available for further experiments the problems may have been overcome and more detailed data may have followed. However, the limited data derived from these experiments suggests that the descending afferents terminate within the deep sensory laminae of the vagal lobe. This was observed solely within the ipsilateral lobe thus confirming the results of injections of tracer into the vagal lobe.

This observation coincides with that found in the Crucian carp. Following extirpation of one inferior lobe, degenerating neurons were observed in the deep sensory layers of this species (Morita et al 1988).

In view of the site of termination of this projection between the sensory input to the vagal lobes and the dendritic field of the motor neurons innervating the oropharyngeal musculature (Chapter 3), a descending influence on feeding reflexes is inferred.

In summary, the experiments reported in this chapter have shown that the ascending projections of the facial and vagal lobes of Cyprinus carpio terminate in the superior secondary gustatory nucleus. This nucleus is topographically organised with respect to the ascending input from the medullary lobes. Thus the somatotopical representation of the primary afferent gustatory input to

the hindbrain is maintained in the higher order projection.

Descending afferents to the medullary lobes have also been shown to be topographically organised. The rostral portions of the nucleus posterior thalami, nucleus lobo-bulbaris and nucleus diffusus lobi lateralis project to the vagal lobes whilst the caudal cells within the nuclei innervate the facial lobe. It has been suggested that the topographical arrangement of central gustatory connections is related to contrasting functional roles of the peripheral gustatory structures.

The descending afferents to the vagal lobe have been shown to terminate in the deep sensory laminae. A projection to this position has been suggested to implicate a role in regulating the motor output of the vagal lobe to the palatal organ.

A SYNOPSIS OF CONCLUSIONS AND SOME FURTHER SPECULATIONS

The gustatory modality of Cyprinus carpio comprises two main components, a "facial" system and a "vagal" system, which have also been termed respectively, the external and internal gustatory systems.

The external component is represented by taste buds situated on the surface of the body and at the outermost locations of the oral cavity, and these are innervated by the facial nerve. In contrast, the internal system comprises of taste buds in the pharyngeal cavity which are supplied predominantly by branches of the vagus nerve.

Although electrophysiological studies of the two systems in Ictalurus show that they receive similar chemosensory inputs (Davenport and Caprio 1982; Kanwal and Caprio 1983), the two systems are functionally distinct components. The results of lesion studies (Atema 1971) have indicated that the external gustatory system is involved in the localisation and selection of foodstuff in the environmental substrate. The internal component is involved in the discrimination of edible from inedible materials actually taken into the mouth and the eventual act of swallowing.

The two systems are also represented in different regions of in the medulla oblongata. Tracer injections

into pharyngeal structures have been shown here to result in labelled elements in the vagal lobe whilst similar injections into structures bearing external taste buds result in facial lobe label.

The experimental studies reported here have shown that wheatgerm agglutinin conjugated to horseradish peroxidase is more efficient in tracing the central and peripheral terminations of primary afferent neurons innervating taste buds of the two systems than unconjugated horseradish peroxidase. In fact, the former is approximately fifty times more efficient.

In addition to the separation in the hindbrain, the regions are structurally different. The vagal lobe is a clearly laminated structure with a superficial sensory region and a deep motor layer. The facial lobe, on the other hand, does not exhibit this laminar organisation and, although it would be incorrect to describe it as disorganised, it has a much more homogeneous structure. These organisational differences between the lobes reflect the contrasting functional requirements of the two taste systems.

The primary afferent neurons from the carp palatal organ have been shown to course into the vagal lobe solely within the deep root. Also, it has been shown that they are topographically organised in the vagal lobe with respect to the taste buds they innervate. Caudal palatal

sites are represented caudally in the vagal lobe and increasingly rostral palatal locations are represented equally rostrally. Medial palatal zones are mapped ventrally whilst lateral zones are represented dorsally in the lobe. A sensory map of the palatal surface coordinates is therefore incorporated into the vagal lobe.

Previous suggestions of such an organisation have come from electrophysiological studies in the goldfish (McClone 1977), and an anatomical study of the goldfish published during the preparation of this thesis has reported equivalent observations (Morita and Finger 1985). Also, the topographical organisation of the vagus nerve input along the rostrocaudal axis of the vagal lobe has been reported in electrophysiological studies of Ictalurus (Kanwal and Caprio 1983).

Investigations of the distribution of taste buds in the palatal epithelium, as judged by labelling the peripheral terminals of the primary afferent neurons, have shown that they are not found homogeneously across the palatal organ. They have a mesh-like organisation in lateral palatal locations whereas in medial palatal sites they are clustered. Similarly, the central terminals of these primary afferents do not display a homogeneous distribution in the vagal lobes. In regions shown to represent lateral palatal organ they are organised in a reticular fashion which resembles the distribution of the peripheral terminals. In ventral vagal lobe locations,

which represent medial palatal organ, the palatal afferents terminate more densely. Consequently, it is tempting to speculate that the sensory map of the palatal surface in the vagal lobe incorporates the heterogeneous distribution of receptors. The sensory map may therefore be a precise representation of the organisation of primary afferent terminals in the palatal organ and also, by association, of the taste bud distribution.

The extensive bed of striated muscle fibres in the palatal organ is innervated by neurons of the dorsal and ventral vagal motor nuclei. This innervation is also topographically organised and has "mapping" relationships which correspond with those of the primary afferent neurons. Thus, the palatal organ is represented as a motor map as well as a sensory map.

The radial organisation of fibres throughout the vagal lobe layers and the observations of similarly restricted labelling of sensory and motor components in the lobe following injections of tracer into limited palatal regions suggest that the two components are mapped in register. This has been confirmed in studies of the goldfish vagal lobe which showed that the intrinsic connections between the sensory and motor layers are topographically organised (Finger 1981b). Thus, the sensory input from the palatal organ and the motor output to its musculature are related such that a stimulus

located at a point on the palatal surface influences only the motor units supplying the stimulated region.

The surfaces opposing the palatal organ are also populated with taste buds and have an underlying matrix of striated muscle fibres. The results of tracer injections into the floor of the anterior pharynx of Cyprinus carpio have shown that medial sites on the surface opposing the rostral region of the palatal organ are represented at ventral locations in the anterior vagal lobe/glossopharyngeal lobe. This coincides with the representation of the anterior midline palatal organ which it opposes. In the goldfish, recently published elegant studies of branches of the vagus nerve which supply the gill arches opposing the lateral regions of the palatal organ have shown that they are also represented in topographically organised sensory and motor maps in the vagal lobe. These maps correspond with those of the opposing palatal surface (Morita and Finger 1985). Thus, matching topographical representations of the opposing surfaces of the entire pharynx appear to be contained in the vagal and glossopharyngeal lobes.

As the maps of opposing pharyngeal surfaces are topographically coincident, and the vagal lobe is radially organised, it seems probable that the motor neurons supplying the musculature at opposing pharyngeal sites will be closely related.

From a functional viewpoint, the sifting and

sorting of material taken into the mouth during feeding (Sibbing 1982) would seem to require an accurate integration of the motor activities of the opposing surfaces. An intimate relationship between the motor neurons supplying the musculature at opposing loci might therefore be expected. It would be interesting to test this hypothesis, perhaps by applying different tracers to the innervations of opposing pharyngeal sites and subsequently assessing the relationships of the differentially-labelled neurons.

The external taste system is also topographically organised. The studies of the carp which I have presented here, and studies of Carassius carassius (Morita et al 1980, 1983) and Ictalurus (Finger 1976) show that a map of external "gustatory space" exists in the facial lobe. The presence of such a map is not surprising in view of the suggestions that the externally-situated taste buds are involved in localising and selecting food sources prior to intake.

The ascending projections from the vagal and facial lobes have been shown to remain topographically discrete in the superior secondary gustatory nucleus of Cyprinus carpio. Although taste responses have been recorded in this nucleus (Marui 1981), its precise role is obscure. However, its hypertrophy in "gustatory" fish is presumably

a reflection of a significant involvement in processing gustatory activity. In the crucian carp, it has been suggested that the separation of vagal and facial inputs is maintained in the ascending projections to the diencephalon from the superior secondary gustatory nucleus (Morita et al 1983). Thus, it appears that the representations of the internal and external gustatory systems remain distinct throughout the ascending gustatory lemniscus. The implication of this organisation is that the two functionally distinct taste systems are maintained as parallel gustatory pathways in the central nervous system. Further support for this view comes from the observations that the descending inputs from the diencephalon to the primary relay nucleus of each pathway are also topographically separable. Also, it has recently been shown in Ictalurus that the intramedullary connections of the facial and vagal lobes are different (Morita and Finger 1985a). It would be useful to re-examine the carp to see if this latter condition were also true in this species.

The question which remains is how does this organisation in teleost fish relate to that found in mammals?

The taste receptors of mammals are found on fungiform, foliate and circumvallate papillae. These papillae are located on the tongue, palate, pharynx,

epiglottis and larynx. Generalising, the chorda tympani which is a branch of the facial nerve innervates taste buds on the rostral two-thirds of the tongue. The glossopharyngeal nerve and the greater superficial petrosal nerve innervate the caudal one-third of the tongue and the palate respectively. The pharynx, epiglottis and larynx are supplied by branches of the vagus nerve.

Unlike the cyprinoid and siluroid fishes, in which the primary sensory nuclei of the facial, glossopharyngeal and vagus nerves are distinct, no such separation exists in mammals. The afferents of these nerves in mammals mainly terminate in the nucleus tractus solitarius (NTS) (Rhoton 1968; Beckstead and Norgren 1979; Ciriello, Hrycyshyn and Calaresu 1981; Contreras, Beckstead and Norgren 1982). However, the gustatory components of these nerves in mammals are found to terminate only within restricted portions of the NTS. For example, in the rat gustatory afferents are found to project to lateral portions only (Hamilton and Norgren 1984) and the same can be said for the chorda tympani of the hamster (Whitehead and Frank 1983). Of particular interest here however are the observations that these gustatory components are topographically organised within the lateral portion of the NTS although there appears to be some overlap. That is, facial nerve branches distribute at rostral levels, vagal branches caudally, and glossopharyngeal components intermediately (Hamilton and Norgren 1984). This

organisation of gustatory afferents within the visceral sensory column of mammals is similar to that observed in cyprinoid and siluroid fishes.

Anatomical studies of ascending projections from the NTS provide further evidence for similarities between "gustatory" fishes and mammals. Projections from the caudal NTS of the rat terminate in the lateral region of the parabrachial nucleus whereas efferents from the rostral NTS terminate in the medial portion of the parabrachial nucleus (Norgren and Leonard 1973; Norgren 1978). It therefore seems that, on the basis of location and input from the primary gustatory nucleus, the parabrachial nucleus (or pontine taste area) may be the mammalian anatomical homologue of the superior secondary gustatory nucleus of fish.

The evidence suggests therefore that the similarities between the central representations of the gustatory systems in cyprinoids or siluroids and those in mammals reflects the presence of a general vertebrate gustatory organisation. The hypertrophy of the systems in cyprinoids and siluroids makes them particularly appropriate for experimental investigation of fundamental questions regarding the gustatory modality in all vertebrates.

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APPENDIX 1.

YOUNG'S FRESHWATER TELEOST SALINE (Young 1933).

5.50g. NaCl

0.14g. KCl

0.16g. $CaCl_2.2H_2O$

Dissolve these in 1000ml. of distilled water.

APPENDIX 2.

PHOSPHATE BUFFER AT pH 7.4

Stock solution A 0.2M NaH_2PO_4 (27.6g/L)

Stock solution B 0.2M Na_2HPO_4 (28.4g/L)

Mix 1 part of solution A to 4 parts of solution B for stock solution of 0.2M phosphate buffer at pH 7.4.

Prior to use add an equal volume of distilled water to provide 0.1M phosphate buffer at pH 7.4.

APPENDIX 3.

GELATIN EMBEDDING MEDIUM

100ml. phosphate buffer at pH 7.4 (Appendix 2).
20g. gelatin powder.

Heat buffer to 50 °C. Whilst stirring add gelatin slowly and continue stirring until the gelatin has dissolved. Cover gelatin solution and place in a water bath maintained at 35 °C.

APPENDIX 4.

GELATIN SUBBING AGENT

150ml. disilled water.

4.5g. gelatin powder.

150ml. 100% ethanol.

Heat the distilled water to 60°C. Add the gelatin powder slowly whilst stirring. When the gelatin has dissolved add the ethanol and continue stirring whilst cooling.

TITLE EXPERIMENTAL STUDIES ON THE ORGANISATION OF THE GUSTATORY SYSTEMS OF THE CARP (CYPRINUS CARPIO)

AUTHOR G. E. BAKER