NEUROSECRETORY CELLS IN THE BUCCAL GANGLIA OF *HELIX POMATIA*

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To My Family

ABSTRACT

NEUROSECRETORY CELLS IN THE BUCCAL GANGLIA OF *HELIX POMATIA*

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The present work herein covers detailed LM studies of Neurosecretory cells by using the Paraldehyde fuchsin (PAF) staining method and the Alcian Blue / Alcian Yellow (AB/AY) histochemical technique in the buccal ganglia of *Helix pomatia*. Two types of Neurosecretory cells, Light green cells and Dark green cells, which had not been reported previously, were identified. Localization and distribution of these cells were mapped from serial sections. They have a rather restricted distribution in the Lateral lobe and Median lobe of the buccal ganglia. Two types of Neurosecretory cells investigated make direct contact with the neural lamella which may constitute neurohaemal

complex. This suggestion was derived from the specific accumulation of stained secretory products in the perikarya abutting to the neural lamella at the site of contact. In Helix, the significance of release areas and the role of somal release of secretory products have been discussed.

In addition, one type of identified Neurosecretory cells has been studied ultrastructurally. Fine cytoplasmic processes penetrating into the neural lamella were also detected in the connective tissue under electron microscope.

Key words : Helix pomatia , buccal ganglia, neurosecretion neurosecretory cells, Molluscs, AB/AY technique.

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ÖZ

HELIX POMATIA'NIN BUCCAL GANGLIYONUNDAKI
NÖROSEKSYON HÜCRELERI

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Bu çalışma, Helix pomatıa'nın buccal gangliyonundaki Nörosekresyon hücrelerinin Paraldehid fuksin (PAF) ve Alcian Blue / Alcian Yellow (AB/AY) methodları ile ısık mikroskobunda yapılan detaylı çalışmaları içerir. Varlıkları önceden rapor edilmemiş iki tip nörosekresyon hücresi, açık yeşil ve koyu yeşil hücreler, bu çalışmada tanımlanmıştır. Bu hücrelerin yerleşim ve dağılım haritası seri kesitler ile belirlenmiştir. Bu hücreler buccal gangliyonunun lateral ve median loblarında oldukça sınırlı bir dağılım gösterirler. Üzerinde çalışılan, bu iki tip

nörosekresyon hücresi nörohemal alanı oluşturan nöral lamellaya direk temas halindedirler. Bu fikir nöral lamellaya bitişik olan hücrelerde temas yerinde boyanmış sekresyon maddesinin özellikle toplanmasından kaynaklandı. Helix'de sekresyon maddesine somal salınımı ve salınım yerlerinin önemi tartışılmıştır.

Ayrıca, belirlenen bir tip nörosekresyon hücreside ultrastrüktürel olarak çalışılmıştır. Bağ dokusunda nöral lamellaya girmiş ince sitoplazmik uzantılarda elektron mikroskopu ile tesbit edilmiştir.

Anahtar kelimeler : Buccal gangliyonu, Helix pomatıa, nörosekresyon, nörosekresyon hücresi, AB/AY tekniği, yumuşakçalar.

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LIST OF SYMBOLS

AB/AY - Alcian blue / Alcian Yellow

App - Appendix

BLC - Blue cells

BLGC - Blue-green cells

BrGC - Bright-green cells

bucc. com - buccal commissure

CH - Gomori's chrome-alum haematoxylin-phloxin

cererbro-buc. con - cerebro-buccal connectives

DGC - Dark green cells

EG - Elementary neurosecretory granules

EM - Electron microscope

GC - Green cells

HE - Ehrlich's hematoxylin with eosin counterstain.

LG - Left Ganglia

LGC - Light green cells

LL - Lateral lobe

LM - Light microscope

LYC - Light-yellow cells

ML - Median lobe

NL - Neuronal lamella

NP - Neuropile

NSC - Neurosecretory Cells

PAF - Paraldehyde fuchsin

RG - Right Ganglia

YGC - Yellow-green cells

LIST OF SYMBOLS FOR FIGURES

Cyt.Pro - Cytoplasmic processes

EG - Elementary granule

H.S - horizontal section

LG - Left ganglion

LL - lateral lobe

Mit - Mitochondria

ML - medial lobe

N - nerve origin

NL - neural lamella

NP - neuropile

Nuc - Nucleus

Pn -Pharyngeal nerve

RER - Rough endoplasmic reticulum

RG - Right ganglion

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All photomicrographes were taken from horizontal sections.

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CHAPTER I

INTRODUCTION

1.1 CONCEPT OF NEUROSECRETION

Neurosecretion is the term used to describe the glandular activities of specialized neurons and is now widely recognized as one of the most important and fundamental phenomena in both vertebrate and invertebrate physiology .

The phenomena of neurosecretion were first put forward by E.Scharrer in 1928 on the large neurons of the preoptic nucleus of a teleost. Scharrer were definitely affirmed the existence of nerve cells with the main attribute of the gland cell, the elaboration of a formed secretory product, in addition to their neuronal characteristics. He suggested the term "neuro glandular cells (Drûnsen nerverzellen)" for these cells, the elaboration phenomenon then being termed as neurosecretion. However, at the beginning the concept of neurosecrection met with considerable opposition (Gabe, 1966). This opposition was eventually ended by the

work of Bargmann (1949) demonstrating of the affinity of the Gomori stains for the product of hypothalamic neurosecretion in vertebrates. As mentioned above neurosecrectory cells (NSC) were first detected in vertebrates (Scharrer, 1928). B.Scharrer (1935, 1936) gave the first description of NSC in Molluscs and Annelieds. Weyer (1935) described the first example of neurosecrection in insects.

NSC have all the characteristics of neurons (Scharrer and Weitzman, 1970). The most important neuronal property of NSC is their capacity for generating and conducting impulses (Gosbee et al, 1968). Most NSC have the same shape as a typical nerve cell, which can be monopolar, bipolar, or multipolar. The size and shape of NSC may vary within very wide limits in different animal species. However, size and shape can not provide a criteria for the identification of the NSC because the ordinary neurons may have very similar morphology. The early historical investigations of NSC in both vertebrates (Scharrer, 1928) and invertebrates (Scharrer, 1937) were carried out by the use of the light microscope (LM). In the identification and classification of NSC, LM is not always adequate. The introduction of the electron microscope (EM) has facilitated the studies on neurosecretion.

NSC are different from ordinary neurons in many respects. Neurons form synapses with other neurons or effector organs. However, axons of NSC end at blood spaces into which they release the neurosecretory material (Scharrer, 1952). Neurotransmitters in ordinary neurons have only to travel across the synaptic cleft (about 20 nm.) , whereas neurohormones produce in NSC may act on much more distant receptors. Classical neurotransmitters convey signals rapidly to elicit responses of extremely short duration (in milliseconds), whereas neurohormones may act over minutes or even hours (Scharrer, 1969). Many recent studies have been concerned with the chemical structure of neurohormons. The first neurosecretory hormones identified were polypeptides, and this fact was regarded as an important feature in distinguishing them from the biogenic amines serving as neurotransmitter. However, Knowles (1965) described a class of neurosecretory elements which were postulated to release amines.

Although the cytoplasmic characteristics of NSC vary considerably both from species to species and in different stages of the secretory cycle in the same animal, there are typical organelles and inclusions which characterise the NSC.

The advent of EM has made possible the precise

neurosecretory products. Neurosecretory material in cells are strictly enclosed in electron-dense, membrane bounded granules that are easily identified and localized by EM. These granules ranging from 100 nm to 300 nm are referred to as "elementary neurosecretory granules " (EG)(Bern, et. al., 1961). The sequence of events in the formation of EG carrying neurosecretory product follows the classical pathway of protein synthesis (Palade, 1975). Neurosecretory product is synthesized in the rough endoplasmic reticulum and packaged by the Golgi apparatus as EG (Pickering, 1976). NSC typically contain EG.

Ultrastructural investigations reveal that neurosecretory product synthesized in the perikarya is transported to the axon terminals within EG. The mechanism by which neurosecretory products is conveyed down the axons to the terminals has been studied in detail in the hypothalamo- neurohypophysial tract (Bargmann, 1949). It is generally believed that release of neurosecretory product takes place from axon terminals within neurohaemal organs. However, there have been reports on somal release (Bayraktaroğlu et . al , 1988).

Several mechanisms have been proposed for neurosecretory discharge. Among minor hypothesis,

exocytosis is the only well-established mechanism of release (Douglas, 1966; Normann, 1974a). Exocytosis involves the fusion of the membrane of EG with that of axon, then the content of EG is released into the extracellular space. Omega figures formed from the joinment of the two membranes can be considered evidence of exocytosis (Normann, 1976).

Classical cytology methods and EM investigations have demonstrated the presence of the essential organelles of both the nerve cells and the gland cells in NSC.

Well-developed endoplasmic reticulum carrying ribosomes is confined to the perikaryon. The smooth endoplasmic reticulum is found together with rough endoplasmic reticulum in the cytoplasm, but it is particularly predominated by the latter in protein/peptide synthesizing NSC. The smooth endoplasmic reticulum is also present in axons (Droz et. al, 1975). Ribosomes independent of the rough endoplasmic reticulum have been observed in NSC (Scharrer, & Brown, 1962).

The presence and functions of Golgi apparatus have been studied by many investigators (Novikoff et al., 1975; Broadwell and Oliver, 1981). The Golgi apparatus found only in the perikaryon is usually associated with Golgi

endoplasmic reticulum lysosomes. Novikoff and his coworkers (1975) believed that EG normally form from the Golgi apparatus and that the Golgi endoplasmic reticulum lysosomes is basically concerned with the production of lysosomes.

The nissl bodies are the earliest known of the cytoplasmic organelles in the NSC. Nissl bodies have been reported in the NSC of many invertebrates, (Gabe, 1966).

The presence of neurofibrils and neurotubules has been shown in a variety of perikarya and axons of NSC (Gabe, 1966).

1.2 PHYLOGENY OF NEUROSECRETORY CELLS

Two schemes have been proposed for the phylogenetic derivation of NSC (review by Gabe, 1966; Lentz, 1968). The investigations of Bargmann (1949) and the Scharrers (1952) showed that NSC are of neuronal origin. From the morphological and functional point of view, NSC may have the significant of epidermal gland cells secondarily incorporated in nerve centres (Clark, 1956). The first scheme is supported by better evidence but the final

solution of the problem requires much additional study (Scharrer and Weitzman,1970).

1.3 TECHNIQUES FOR DETECTION OF NEUROSECRETORY CELLS

The work in detecting of NSC has been made relatively easy by applying staining techniques. Heidenhain's azan, paraldehyde function (PAF), Ehrlich's haematoxylin with eosin (alcoholic) counter stain (CH), Masson's trichrome stain were the original methods used in demonstrating NSC in both vertebrates and invertebrates. (Gabe, 1966)

The development of the alcian-blue/alcian-yellow (AB/AY) technique (Peute and Van de Karner, 1967) has added another feature to cell type identification. By regulation of the pH of AB/AY dye solutions, the neurosecretory materials are stained differentially, thus indicating the various types of NSC and secretory products within the system. The theoretical basis for this differentiation is found in the selective electrostatic binding affinities exhibited by the two Alcian dyes. Alcian blue is applied at pH 1.0 to bind strongly acidic sites, then Alcian yellow is applied at pH 2.5 to bind weakly acidic sites that have been found in the material by the prior oxidation of

aldehyde and hydroxyl groups. The ratio of strong to weak acidic sites is thought to determine the staining character exhibited by the cells ranging in coloration from blue - green to yellow.

The Dark green cells (DGC) which have a high affinity for alcian blue contain large amounts of cystine or cysteine. The yellow cells (YC) that have little or none affinity for alcian blue have a high affinity for alcian yellow and the light green cells (LGC) might have equal ratio of both strong and weak acidic groups (Swindale and Benjamin 1976). With this method various NSC types can be recognized within the class of "Gomori-positive" cells. Cell types stain in different shades of green, depending on the chemical composition of their contents (Van minnen et al., 1977). With AB/AY technique, "Gomori-negative" cells can also be distinguished. The AB/AY technique used proved to be superior to classical neurosecretory stains used.

The histochemical differences among the cell types were confirmed at the ultrastructural level, as the various neurosecretory materials are packaged in EG of different size and appearance (Wandelaar - Bonga 1970; Boer et al. 1977).

1.4 ORGANIZATION OF NEUROSECRETORY SYSTEMS

1.4.1 NEUROSECRETORY CELLS IN VERTEBRATES

NSC in vertebrate occur aggregate as ganglionic nuclei, whereas in invertebrate they either exist as groups of cells or show scattered distribution.In higher vertebrates, NSC are found in the hypothalamus of the brain. The hypothalamus has a role in the control of the pituitary gland secretion. Bargmann (1949) traced the neurosecretory fibre tracts connecting the hypothalamic cell bodies, where the material is synthesized, with the posterior pituitary where it is released into the circulation. These cells constitute the magnocellular component of the nuclei supraopticus and paraventricularis of the mammalian hypothalamus which in fishes and amphibians are represented by the singular nucleus preopticus. Hypothalamic neuron-derived hormones oxytocin and vasopressin are transported by intraaxonal transport to the posterior pituitary. Other NSC in the hypothalamus send their axon endings, upon capillaries in the anterior part of the infundibular stalk, the median eminence. These capillaries pass to the anterior pituitary, carrying blood containing stimulatory or inhibitory neurosecretory factors which control the release and the production of anterior pituitary hormones.

1.4.2 NEUROSECRETORY CELLS IN INVERTEBRATES

NSC in invertebrate are often more widely distributed throughout the nervous system, but there is a tendency to aggregate the cells in the ganglia. In the less organized invertebrates epithelial endocrine glands are apparently absent. Neurosecretions therefore are the only hormonal coordinators.

In Hydra (Coelenterate) NSC are present in the nerve net and release their contents into the mesoglea (Burnett, and Diehl, 1964).

Turbellaria have many NSC in the ventral part of the brain, increasing in number in the more posterior regions (Lender, and Klein, 1961).

Within the Nematoda, Ascaris lumbricoides has NSC in the lateral ganglia on the anterior nerve ring and Phocanema decipiens has similar cells in the dorsal and ventral ganglia (Davey, 1965).

In polychaetes and oligochaetes, groups of scattered NSC are found in the brain, or supra-oesophageal ganglion, and in the ganglia of the ventral chain (Gabe, 1966). In polychaetes annelid, Nephtys, four types of NSC with

distinctive cytoplasmic inclusions have been described (Zahid and Golding, 1975). "a" cells are located in groups but "c" and "d" cells have a scattered distribution in the cerebral ganglia "b" cells are present as a single pair.

The insects neuroendocrine system has four major components : groups of NSC in the brain, the corpora cardiaca, the corpora allata, and the thoracic glands or their equivalents. The corpora allata are connected by nerves to the corpora cardiaca and to the suboesophageal ganglion. The thoracic glands are innervated variously from the suboesophageal ganglia and thoracic ganglia. The corpora cardiaca are usually inclose association with the dorsal heart (aorta) and are still often referred to as oesophageal ganglia. The corpora cardiaca produce their own intrinsic hormones, but their major function in most insects is to store and release neurosecretory hormones from the brain. The cerebral NSC are in two groups, in each half of the pars intercerebralis, region of the forebrain. The corpora cardiaca receive axon from the cerebral NSC (Highnam, 1965).

In crustaceans, NSC are found in the eyestalks, brain, suboesephageal ganglion and throughout the remainder of the central nervous system (Gabe,1966).NSC are within the optic ganglia which are called the x-organs (Highnam, 1965).

1.4.3 NEUROSECRETORY CELLS IN MOLLUSCS

The presence of NSC has been demonstrated in all groups of molluscs (Gabe 1966; Golding,1974; Boer and joosse, 1975). The most extensively studied neurosectory systems are of the two orders of pulmonates, namely Basommatophora and Stylommatophora. The nomenclature of NSC used in these groups, is based on the location and staining properties of NSC.

In Lymnaea stagnalis, seven different types of Gomori positive NSC have been distinguished in the central ganglia (Wandelaar - Bonga, 1970). In addition, four of types of phloxinopilic Gomori negative cells occur, of which three stains with alcian yellow.

In the cerebral ganglia, two types of Gomori positive cells LGC, Bright Green-cells (BrGC) and two types of Gomori negative cells are present (Joosse, 1964).

Two large groups of LGC occur in the medio- and latero-dorsal parts of the ganglia, under the endocrine medio- and latero-dorsal bodies. The axon of the LGC form a main bundle which runs into the median lip nerve which serve as a neurohaemal area (Joosse and Greaets, 1969).

The Gomori positive BrGC are relatively small and occur in two groups of 10-25 cells in each cerebral ganglion. One group is located near the medio-dorsal cells between the anterior lobe and cerebral commissure; the other lies adjacent to the neuropile between the medio- and latero- dorsal cells. Their axon could not be traced to their terminals and consequently their neurohaemal area is not known.

The first type of Gomori negative cells are the phloxinophilic caudo-dorsal cells. They are situated caudo-dorsally in groups of 20-40 cells in the left, of 50-100 cells in the right ganglion and their axon run into cerebral commissure, the neurohaemal area of these cells. The caudo-dorsal cells are not stained by AB/AY.

Other Gomori negative cells occur in two small groups of 1-3 cells. They are located near the origin of the statocyst nerve (Boer, 1965). They are stained intensely yellow with AB/AY.

It appears that all ganglia of Lymnaea stagnalis possess NSC with the exception of the buccal and the pedal ganglia.

In the visceral ring, which consists of the paired

pleural and parietal ganglia and the single visceral ganglion, two types of Gomori positive cells [DGC and yellow green cells (YGC)] and two types of Gomori negative cells [light yellow cells (LYC) and yellow cells (YC)] occur. Their axon do not form major tracts but run individually or in small group into the peripheries of the connectives and nerves of the ring and also large area of the perineurium (Hekstra and Lever, 1960; Wandelaar-Bonga, 1971; Swindale and Benjamin, 1976).

In Melanpus bidentatus (Pulmonate; Basommatophora), seven to eight AB/AY NSC types occur within each cerebral ganglion. A group of LGC is situated mediodorsally within the cerebral ganglia. DGC of the cerebral ganglia can be divided into two size classes. Medium size DGC are lied medioventral to the root of the cerebro-pleural connective and small DGC are located medial to the tentacular nerve root. Two dark yellow cells (DYC) occur in each posterio dorsal edge of the pars dorsalis, and the other lying just medial to the later lobe. There is also a cluster of YC positioned medioventrally within the pars dorsalis.

Two to five DGC lie within the anterodorsal region of each pleural ganglia.

The pedal ganglia contain both DGC and DYC. The DGC

occur as two separate groups, one forming a light cluster in the latero-ventral region of each ganglion and the other located along the medial edge of each ganglion (adjacent to the pedal commissure). The pedal ganglion YC occur near the latero-ventral DGC.

Green cells (GC) are situated in the visceral ganglion near the posterior edge of the parieto-visceral connective and the other GC in the laterodorsal region of the right parietal ganglion. Blue green cells (BlGC) are located within the right parietal ganglion. Medium and large YGC are also present within the parietal and visceral ganglia.

The present of many NSC types in the central nervous system of stylommatophora was revealed by LM studies (Kuhlmann and Nolte, 1964). In contrast to lymnaea, there are relatively less reports about the neuroendocrine system of Helix (Whittle, 1978; Kai-Kai and Kerkut, 1979; Wijdenes et al, 1980). These authors studied the NSC with PAF, AB/AY. Most of these studies were done by LM. However, more recent years, EM studies have commenced the detailed exploration of the NSC (Whittle, 1978; Kai-Kai and Kerkut, 1979; Wijdenes et al, 1980; Bayraktaroğlu et al, 1988).

Whittle (1978) studied the cerebral ganglia of Helix pomatıa by using PAF staining and by EM. He has categorized

cell types from A to M on the basis of their size of inclusions.

NSC have been identified in the central nervous system of H. aspersa with the AB/AY technique (Kai-Kai and Kerkut, 1979). These authors distinguished between DGC, LGC and YC.

Similarly, the AB/AY staining technique was used by Wijdenes et al, (1980) to demonstrate NSC in the ganglia of H.aspersa, Deroceras reticulatum and Arion hortensis. These authors described variety of cell types, distinguished by their location and staining affinity. They have reported seven types of NSC stained which are GC, LGC, YGC, YC, DGC, BIC (blue cells) with AB/AY method are located in the cerebral ganglia and three NSC types were distinguished in the visceral complex (pleural, parietal, visceral). One of the two BIGC is located in the caudal part of the right parietal ganglion, the other in the medio-caudal area of the visceral ganglion. LGC are located in the right latero-caudal part of the visceral ganglion. The BIGC occur scattered throughout the right half of the visceral ganglion.

It is difficult to establish the equivalence of NSC types described by the above authors in the central nervous system of Helix.

NSC in the buccal ganglia of Helix has been reported only by Wijdenes et al, (1980). They have reported the presence of 8-10 BrGC.

1.4.4 NEUROHAEMAL ORGANS

The neurosecretory pathways are characterized by terminations onto organs which are in immediate contact with large blood vessels, blood spaces or the general body cavity. These organs containing neurosecretory axon terminations are designated as neurohaemal organs. These organs may be concerned in the discharge into the circulation of the active substances elaborated in the perikarya and carried along axon paths (Gabe, 1966).

Vertebrates have the well defined neurohaemal organs, the neurohypophysis and urophysis. The former consists of NSC in the hypothalamus and their axons and axon terminals in the neurohypophysis. The latter contains axon terminals in the caudal region of fishes (Bern and Takasugi, 1962).

In insects the corpus cardiaca with axons from NSC in the brain from the most important neurohaemal organs which involve the storage and release neurohormones.

There are three major neurohaemal organs in crustaceans, namely, the sinus gland, the post commissural organs and the pericardial organs. The sinus glands receive axons from the ganglionic x-organ, the post-commissural organ from the brain and pericardial organs from the thoracic ganglia.

Unlike vertebrates, insects and crustaceans, the molluscs have no special neurohaemal organs. The NSC terminate and release neurosecretory material onto the surface of the cerebral commissure, connectives, nerves (Simpson, 1969; Wandelaar - Bonga, 1970) and ganglia (Fernandez and Fernandez, 1972; Swindale and Benjamin, 1976). The surface of the above organs are called the neurohaemal areas.

In Basommatophora, neurohaemal areas of NSC are located in the peripheries of commissures, connective and nerves where the neurosecretory material is released by exocytosis from axon terminals into the haemolymph (Wandelaar - Bonga, 1970; Boer and Joosse, 1975; Roubos and Wal-Divendal, 1980). In Stylommatophora, conversely little is known about the location of the neurohaemal areas of the NSC (Whittle, 1978; Joosse, 1969; Wijdenes et all. , 1980). Because there is no certain neurohaemal areas where release of neurosecretory products takes place. Release from

perikarya has been shown to occur in the central nervous system in Helix aspersa and Helix pomatia (Bayraktaroğlu, 1984).

The aim of the present study is to localize and classify the NSC by using the AB/AY histochemical technique in the buccal ganglia of Helix. In this study, Helix pomatıa is mainly used. Two types of NSC, LGC and DGC, which had not been reported, were identified, and their specific locations and distribution were mapped. Electron microscopical studies were also undertaken to observe NSC and their fine cytoplasmic processes in the NL indicating release areas.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

Paraffin, potassium permanganate, phosphotungstic acid, basic fuchsin, hydrochloric acid (concentrated), paraldehyde, absolute ethanol, sulphuric acid (concentrated), toluidine blue, promine, borax, canada balsam, acetic acid, sodium metabisulphite, phloxin B, formaldehyde, gelatin, xylol, chloroform, propylene oxide were purchased from Merck. Alcian Blue 8GX and Alcian yellow GXS were obtained from sigma chemical company, Saint Louis, Missouri, USA. Lead citrate uranyl acetate,formvor, and as embedding media-epoxy kits were purchased from Polysciences Ltd..

2.2.1 MAINTENANCE OF ANIMALS

Specimen of living, adult Helix pomatia were locally collected from the field and kept until used in a large plastic container (30cm x 25cm x 20cm). Soil was distributed over the floor of the container. The container was kept moist by sprinkling with water and the snails were fed with lettuce leaves. The container was kept moist by sprinkling water and the snails were fed with lettuce leaves. The container was kept in the laboratory at room temperature away from direct sunlight.

2.2.2 CLASSIFICATION OF ANIMAL

Phylum	: Mollusca
Class	: Gastropoda
Subclass	: Pulmonata
Order	: Stylommatophora
Family	: Helicidae
Genus	: Helix
Species	: Helix pomatia

2.2.3 DISSECTION OF ANIMALS

As the snails were active, the shell and its contained tissues were separated from the rest of the body by gently lifting the shell as the snail progressed along and by cutting through the connecting part with a pair of scissors. Having removed the shell and its contents, the body was then pinned dorsal side up through the margins of the foot onto a wax dish. An incision was made dorsally into the body wall starting at the existing cut and passing forward to the mouth along the median axis of the snail. The resulting flaps of skin were then turned out and pinned, any restraining muscles being cut as they were drawn back. The buccal ganglia were methodically dissected out. The whole procedure usually took approximately five minutes. The excised ganglia were immediately fixed as required either for light microscopy or electron microscopy studies.

2.3 METHODS

2.3.1 TECHNIQUES FOR LIGHT MICROSCOPY

Bouin's fixative (Luna, 1968) was used throughout this work. In the use of fixative, the removal of picric acid

from tissues was most essential in order to insure proper staining of the tissue sections. The basic procedure was as follows:

a) Fixation :

Tissues were fixed with aqueous Bouin's fixative for at least 24 hours at room temperature, washed in 70% alcohol.

b) Dehydration :

Fixed tissues were dehydrated in the following manner at room temperature.

	Duration
70% alcohol (3 changes)	45 mins.
90% alcohol (3 changes)	45 mins.
100% alcohol (3 changes)	45 mins.
100% alcohol (2 changes)	30 mins.

c) Clearing :

Chloroform was used with two changes for 15 minutes as a clearing reagent after dehydration.

d) Impregnation :

After pouring off the clearing reagent, the tissue was treated with xylene for 3-5 minutes. Then xylene was poured off and equal amounts of molten wax and xylene were added to the specimen tube containing the tissue and kept at 60°C. About an hour later, the molten wax was replaced with fresh molten wax (Paraplast: M.P. 56-57°C).

e) Embedding:

The impregnated tissue was then embedded in molten wax according to the require orientation which were horizontal, transverse, and sagittal plane of the ganglia.

f) Sectioning :

Serial sections, 5,6,7 µm thick were cut on a American Rotary Microtome and mounted on clean, chrome alum-gelatin coated glass slides. Slides were kept on the hotplate at least overnight to ensure good adhesion of the sections.

g) Staining :

After the dehydration of sections, they were stained with the AB/AY technique (Ridgway, 1987) and paraldehyde fuchsin (PAF) method (Gabe,1966).

The staining protocol of AB/AY technique was as follows :

1. Deparaffinize and bring sections to water
2. Hydrolyze in 1N HCl for 6 min. at 60°C (to eliminate background RNA staining)
3. Oxidize for 3 min. in acid permanganate solution (0.25% $KMNO_4$, 0.5% H_2So_4)
4. Bleach in 2% sodium metabisulphite or 2% oxalic acid and rinse in tap water.
5. Rinse in pH 1.0 acetic acid HCl (3% acetic acid with pH adjusted using concentrated HCl)
6. Stain for 30-40 min. in a filtered 0.5% aqueous solution of alcian blue 86x in pH 1.0 acetic acid-Hcl, rinse in pH 1.0 acetic acid-Hcl
7. Rinse in 3% acetic acid (pH 2.5), stain for 30-40 min. in a filtered 0.5% aqueous solution of alcian yellow in 3% acetic acid (pH 2.5), rinse in 3% acetic acid,rinse in tap water.
8. Stain for 10-15 sec.in a 1% aqueous solution of phloxin B, rinse in tap water.
9. Treat in 5% phosphotungstic acid for 3-5 min. rinse in running tap water for 5 min.
10. Differentiate phloxin in 70% alcohol for 30-60 sec.
11. Dehydrate rapidly in alcohol to xylene and mount in canada balsam.

The staining protocol of PAF was as follows :

1. Deparaffinize and bring sections to water
2. Oxidize with Gomori's permanganate of potassium-sulphuric acid mixture for about 1 min.
3. Rinse rapidly in tap water and decolorize with 3 or 5% sodium bisulphite.
4. Rinse briefly in tap water and stain with the dilute acetified paraldehyde fuchsin solution for from 3 to 5 mins.
5. Rinse in tap water and differentiate with absolute alcohol or 35% hydrochloric acid has been added (10-20) sec. usually is sufficient.
6. Wash in tap water, counter stain and/or dehydrate, clear and mount in balsam.

h) Photography :

Sections were examined with an olympus BH2 LM. Photomicrographs were taken with an Axioghot Carll Zeiss photomicroscope using Kodak Gold film. Developments and printings of the films were done by a commercial firm, SANFO.

2.3.2 TECHNIQUES FOR ELECTRON MICROSCOPY

a. Fixation :

Excised tissues from adult snails (Helix pomatıa) were fixed in the 1% O_sO_4 for 2 hours at 0-4°C .

b. Dehydration :

Fixed tissues were dehydrated in the following manner at room temperature.

		Duration
30% alcohol	(2 changes)	15 mins.
50% alcohol	(2 changes)	15 mins.
70% alcohol	(2 changes)	15 mins.
90% alcohol	(2 changes)	30 mins.
Absolute alcohol	(2 changes)	30 mins.
Propylene oxide	(2 changes)	30 mins

c. Infiltration :

	Duration
Propylene oxide + Epon 812 (2 changes)(room temp.)	2 hours
Epon mixture (at 35°C)	12 hours
Epon mixture (at 45°C)	12 hours

d. Embedding :

The infiltrated tissues in the above stage were embedded in fresh epon mixture at 60°C for at least 14 hours. The epon mixture was prepared according to the procedure of Luft(1961), (see App).

e. Sectioning :

Glass knives with an angle of 45° were made on an LKB knife maker. Thin and semithin sections were cut on a supernova ultramicrotome and floated off onto distilled water. Semithin sections (1µm thick) were mounted on glass slides and stained with a hot 1% solution of toluidine blue in a 1% solution of Borax for examination with the LM. thin sections, ranging from 500°A to 700°A in thickness, were stretched with chloroform vapour before being picked up onto copper grides with formvor support films (0.3% formvor in chloroform).

f. Staining :

Thin sections on copper grids were stained according to Reynolds(1963). Sections were stained in the following order.

	Duration
Aqueous saturated uranyl acetate solution (in the dark)	1 hour
Rinse with distilled water	12 mins.
Lead citrate (in a petri dish,using NaOH granules to absorb Co_2 in the air)	30 mins.

g. Photography :

Sections were examined in EM 9 SA zeiss EM. operated at 80 KV. Photomicrographs were taken on AGFA EM. films (polyester base) and printed using IIford photographic papers.

CHAPTER III

RESULTS

3.1 LIGHT MICROSCOPE OBSERVATION

3.1.1 GENERAL ANATOMY OF BUCCAL GANGLIA.

The nervous system of Helix consists of typically paired cerebral, buccal, pedal, pleural, parietal ganglia and one visceral ganglion which actually represents subintestinal and visceral ganglia fused. Paired cerebral buccal and pedal ganglia are connected by commissures named with related ganglia. Cerebral ganglia are connected with buccal, pleural and pedal ganglia with associated connectives named with the respected ganglia.

The paired buccal ganglia is situated below the oesophagus and connected to the cerebral ganglia. The buccal ganglia with about 1300 x 450 x 570 µm in size have the right and left, cerebro-buccal connectives (cerebro-buc.con.) with which join to the brain (cerebral ganglia). The two ganglia each with about 300 µm in diameter are attached with a single buccal commissure (buc.com) with 0.2

mm long. The structure of the buccal ganglia of Helix was described in detail by Kunze (1921), which is typical of molluscan ganglia.

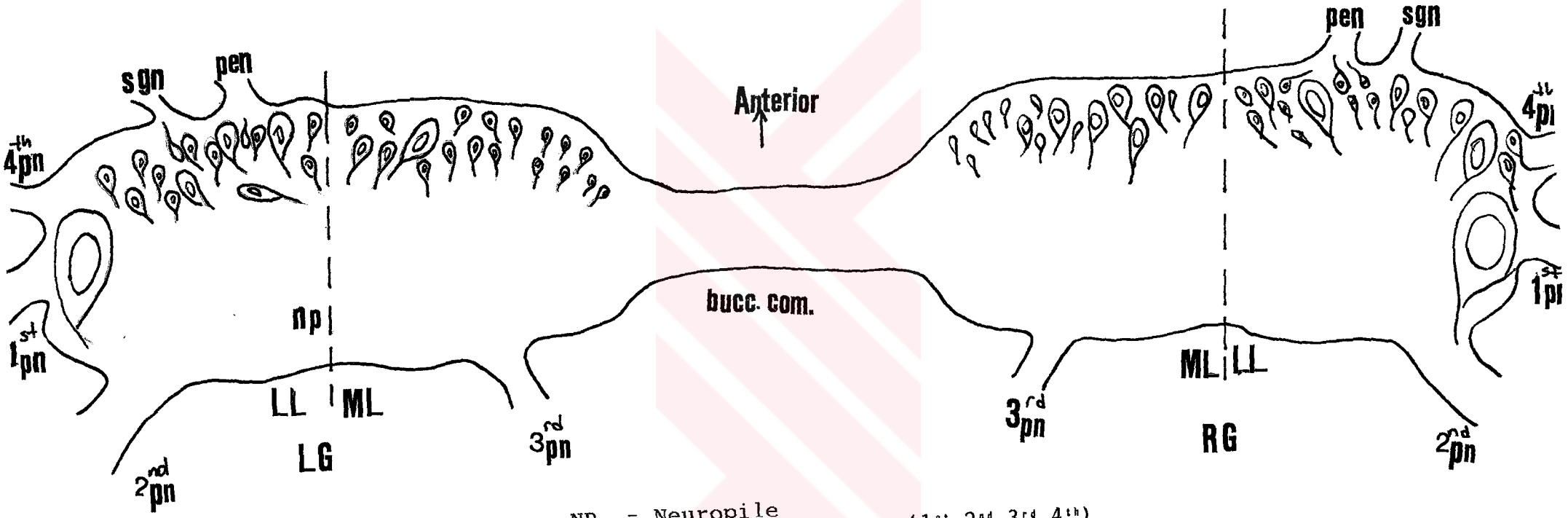
The right and left buccal ganglia are symmetrical. Each ganglia has two lobes, medial lobe (ML) and lateral lobe (LL) (Altrup, 1987). These lobes are separated from each other with a shallow groove (Fig. 2,8,10,13). The ML is close to the buc. com. and 3rd pharyngeal nerve originates from the posterior of it. The LL is situated laterally and give rise to the 2nd , 1st , 4th pharyngeal nerves, the salivary gland nerve and the posterior oesophagial nerves from posterior to anterior (Fig. 1).

The cells of the buccal ganglia were observed unevenly distributed in the periphery of the ganglia. Cells are localized particularly on the surface areas of dorsal, lateral, ventral and anterior regions. The posterior regions do not contain cell groups. Cells which are widely different in size, ranging from 9 µm to 170 µm in diameter including giant neurons from a cortex around the ganglia (Fig. 1,2). The centres of the ganglia called neuropile (NP) contain nerve axon tracts (Figs. 1,2).

3.1.2 IDENTIFICATION OF NEUROSECRETORY CELLS IN THE BUCCAL GANGLIA.

NSC types were distinguished among the ordinary neurons in the buccal ganglia at the LM level. They were easily identified due to their staining properties with different histochemical techniques. Staining affinity of the cell cytoplasm has been basically utilized as one of criteria in the identification of NSC. In this study, PAF and AB/AY staining techniques were mainly used.

In sections stained with PAF, NSC were observed in deep purple colour (Figs. 2, 3). The dark purple stained cells were called PAF (+ve) NSC. The nuclei of these cells do not react, but the cytoplasms only take up the stain. The appearance of nuclei is not different in all cell types observed in sections stained with various methods.



NP = Neuropile
pn = pharyngeal nerves (1^{st}, 2^{nd}, 3^{rd}, 4^{th})
sgn = salivary gland nerve
pen = posterior oesophageal nerve

Fig. 1 Diagram of the buccal ganglia with origines of main buccal nerves Helix pomatia.

Fig. 2. Shows a group of PAF (+ve) NSC with dark purple cytoplasm in the lateral lobe of the ganglia.Inset shows the same group of cells in the next section.

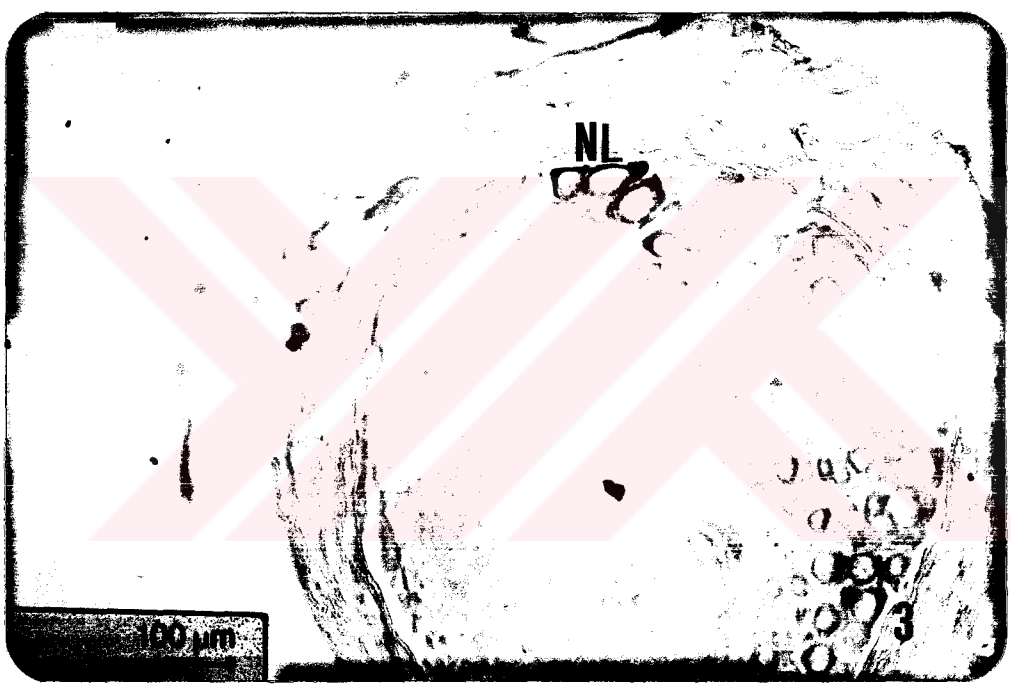


Fig. 3. Nicely stained NSC with PAF can be easily noticed at the periphery of the ganglia adjacent to the NL.

The NSC were stained either dark green or light green in section prepared with AB/AY method (Fig. 4). These cells are named as dark green cells (DGC) and light green cells (LGC) respectively (see 3.1.4 and 3.1.5).

In epoxy sections stained with toluidine blue, some NSC were stained with dark blue (Fig. 5), others were stained light blue color (Fig. 6).

3.1.3 NEUROSECRETORY CELL TYPES

In the present study, two main cell types in the buccal ganglia of Helix pomatia, have been differentiated among nerve cells recognizable at the LM level. They are designated as DGC and LGC on the basis of their tinctorial properties with the AB/AY method.

3.1.3.1 DARK GREEN CELLS

In sections stained with AB/AY technique, some of the stained cells were observed with dark green perikarya. They have been classified as DGC.

DGC are not large in comparison with other neurons,

except giant neurons, in the buccal ganglia, with a mean dimensions of approximately 10 x 60 µm (Figs. 7,8,9). A few giant neurons with about 100 µm to 150 µm in diameter were observed among ordinary neurons and NSC (Fig. 4). DGC appear to have elongated, oval or spherical cell bodies.

The nuclei of these cells with diameter ≈30 µm also are elongated, oval or spherical in appearance and contain certain little chromatin material and stain lightly pink color (Fig. 7,8). The nucleus of DGC characteristically occupies the most of the perikarya, leaving a very thin layer around the former (Fig. 7,8,9,15). The thin layer of the perikarya is filled by secretory products which tend to accumulate to the site associated with the NL (Fig. 4,7).

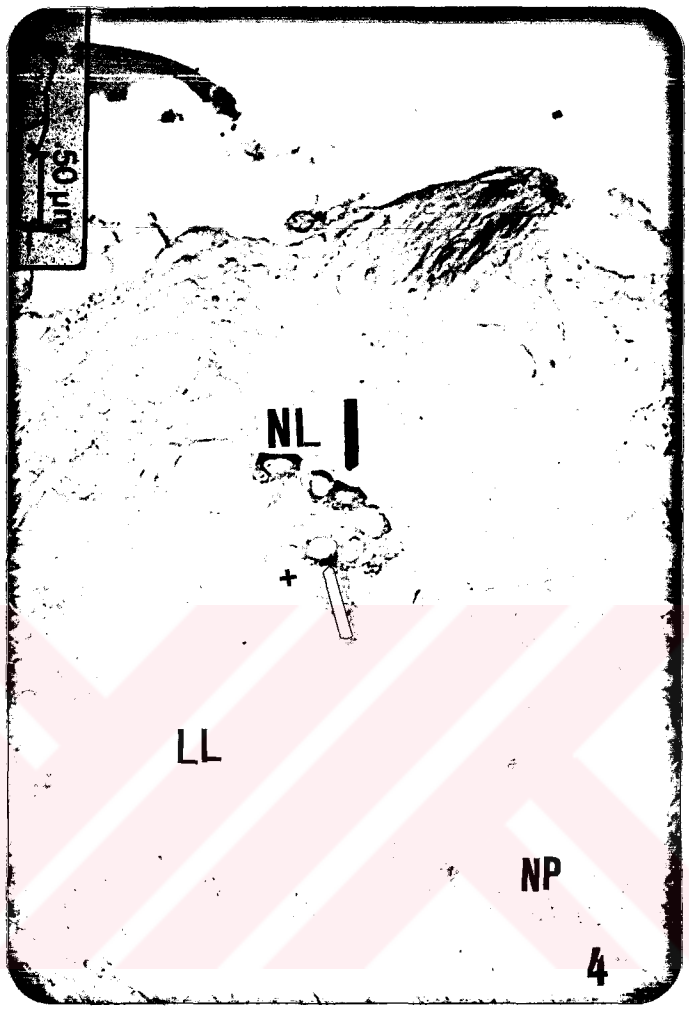


Fig. 4. Dark arrows show DGC adjacent to the NL, white arrows show LGC further from the NL. Notice the arrow head and asterix indicating axons of DGC and a giant neuron directing into the neuropile. Lateral lobe of the right ganglion, AB/AY.

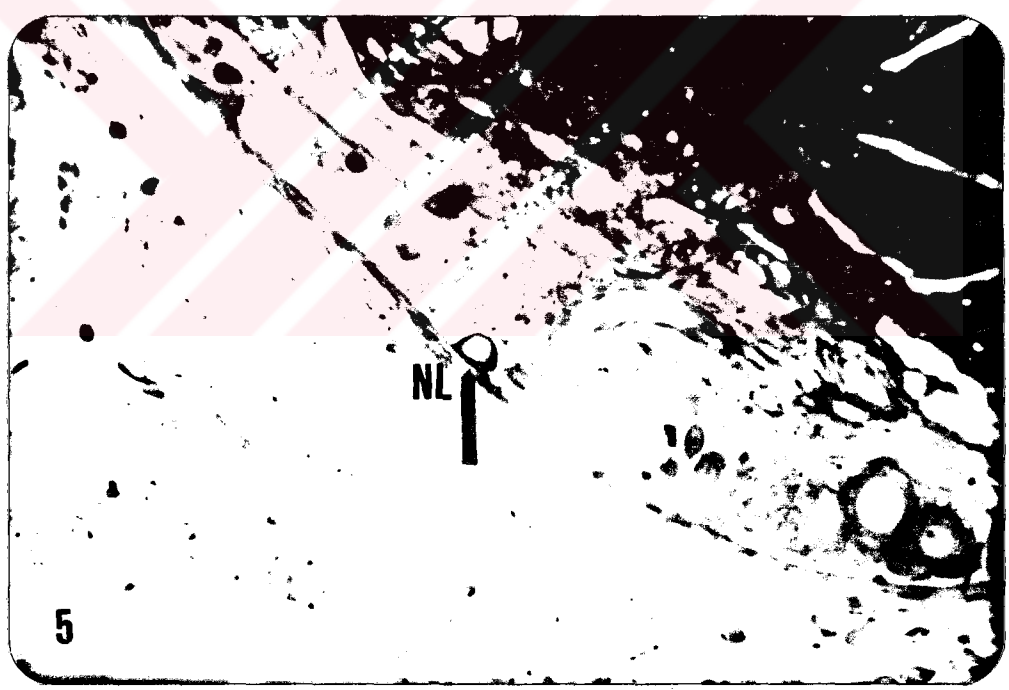


Fig. 5. An arrow shows a dark stained NSC in the epoxy section. Notice that the cell is in direct contact with the NL. Toluidine blue.

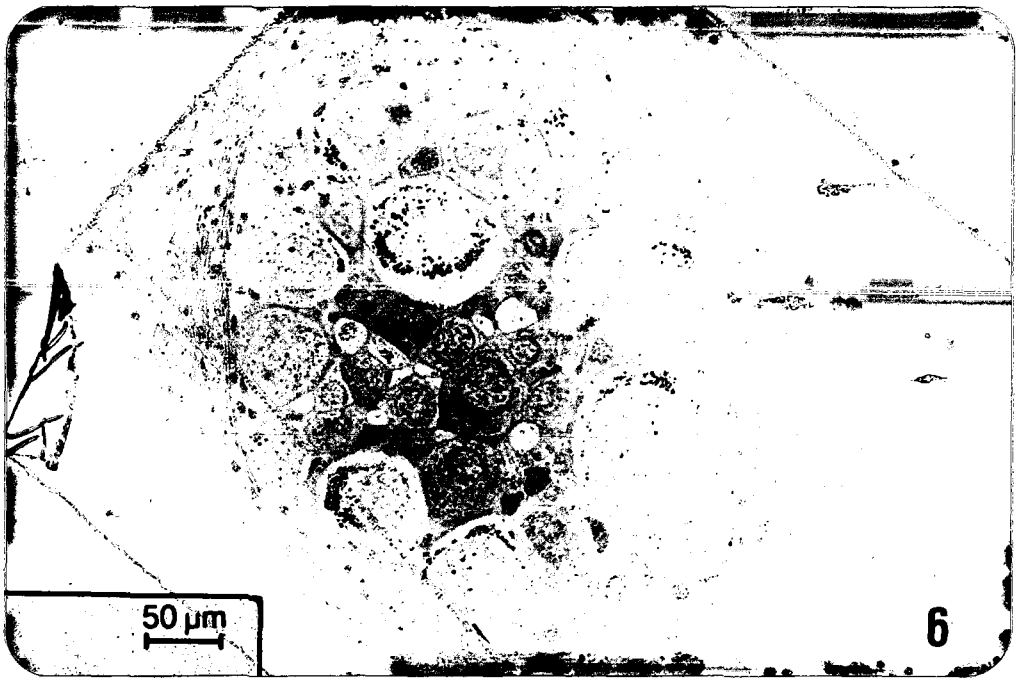


Fig.6. Light blue stained possible NSC located (asterix) among ordinary neurons and giant neurons. Toluidine blue.

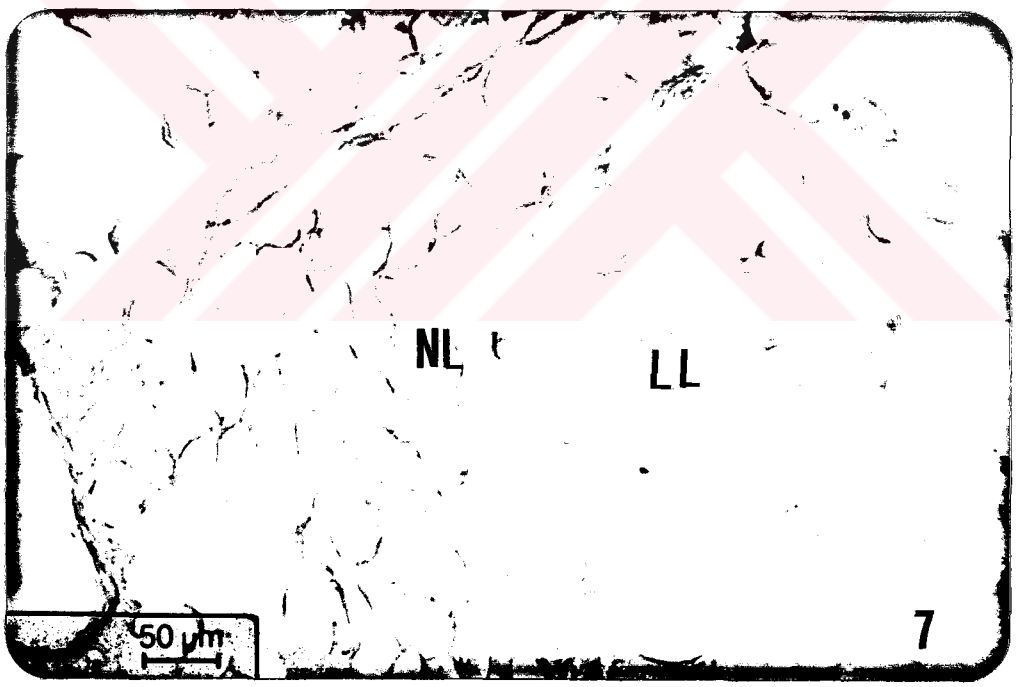


Fig.7. Shows a single DGC in the LL (arrow). Notice the dark green stained cytoplasm abutting to the NL. AB/AY

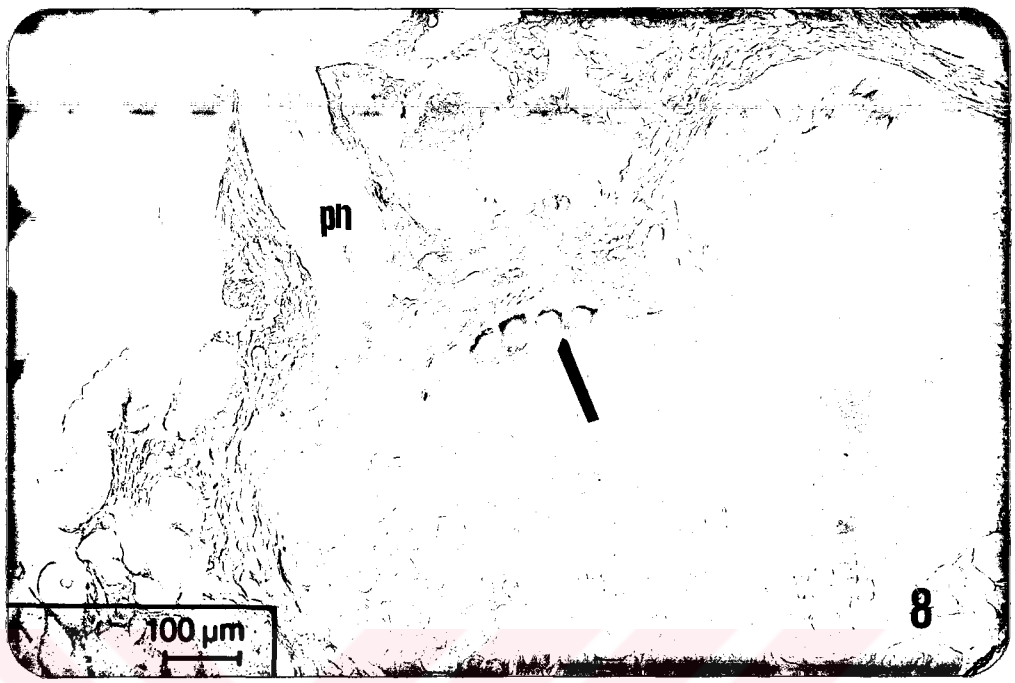


Fig.8. Showing four of DGC in the arch of LL close to pharyngeal nerve. Arrow head depicts the axon hillock of a DGC toward to the NP. AB/AY

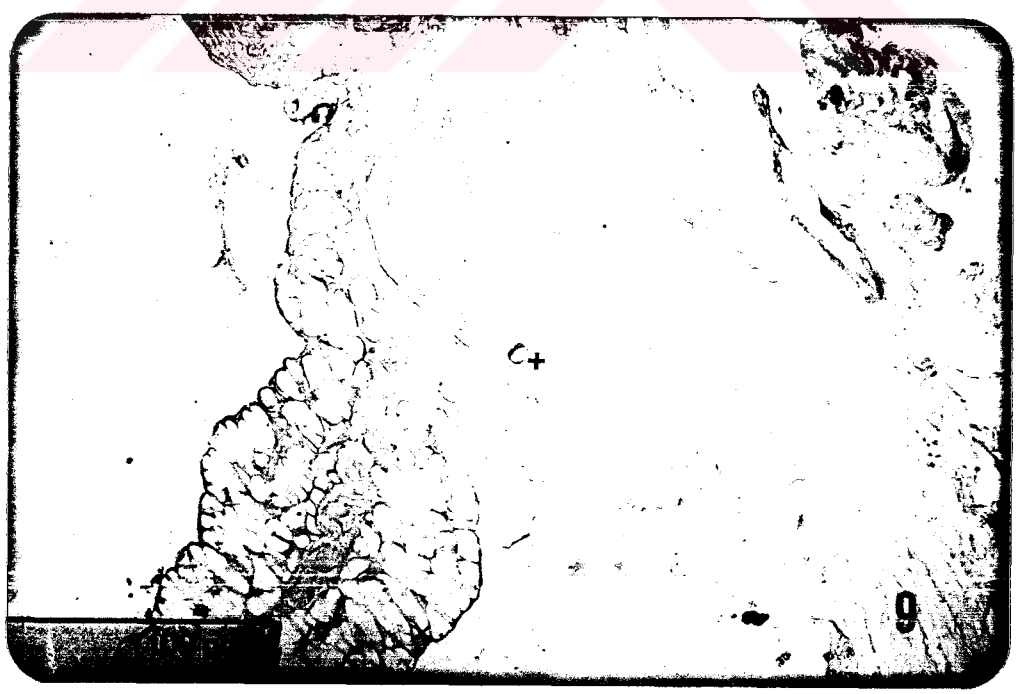


Fig.9. Shows one DGC located in the right ganglion which does not make direct contact with the NL. AB/AY

The axons of DGC can not be traced for a long distance, since they often intermingle with other fibres. In a few cases, the axons of DGC could be observed for a short distance, later extending towards to the NP (Figs. 4,8). Cytoplasmic extensions or processes have not observed at the LM level. They have seen in the ultrastructural observations (see 3.2.2).

In most cases, DGC perikarya have observed making direct contact with the NL (Figs.4,7,8,10,11). In a few cases, DGC located in the ganglia do not make direct contact with the lamella. They are situated away from the NL by about 20 µm distance (Fig. 9) .

3.1.3.2 LIGHT GREEN CELLS

In sections treated with AB/AY, some cells were found to have light green perikarya. These NSC have been classified as LGC (Figs. 5,12)

LGC have elongated, circular or oval shapes, with mean dimensions approximately 20 µm to 70 µm. Like DGC, LGC also have big nuclei with 20 µm in diameter, which occupy the most of the perikaya (Figs. 4,10). The axons of LGC could not be traced in paraffin sections.

Most of the observed LGC do not make direct contact with the NL, but may be separated from the latter by about 20 µm to 50 µm (Fig.10). In rare cases, LGC are found closely associated with the NL (Figs. 11,12).

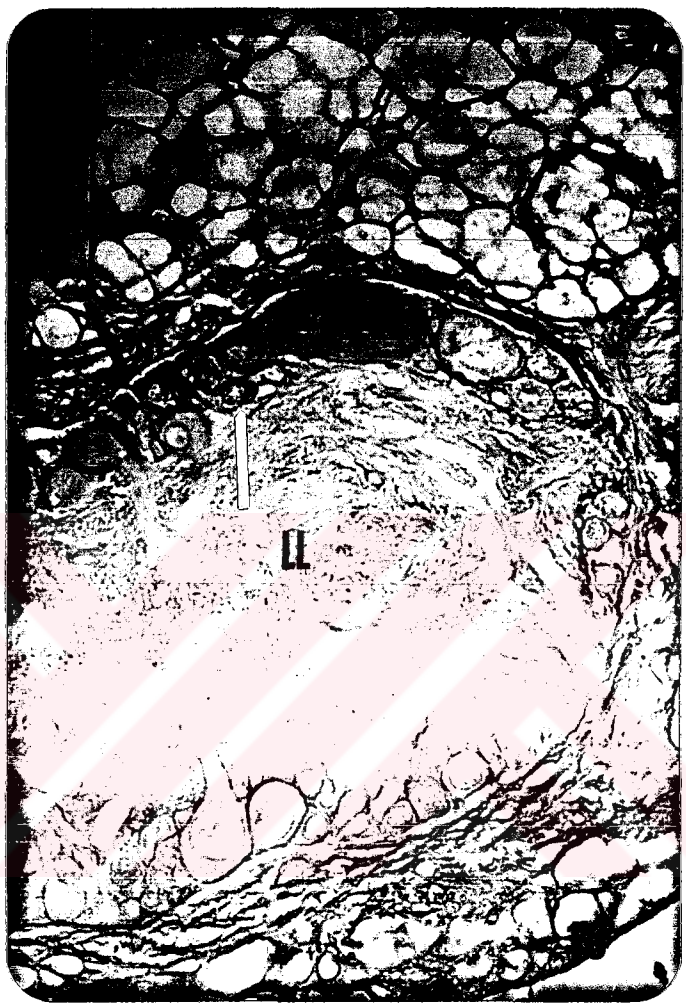


Fig.10 A group of DGC (black arrows) and LGC (white arrows) are seen in the LL. LGC are located further away from the NL, the peripheral region of the lobe. AB/AY

Fig. 11 Shows a group of DGC and LGC in the serial sections. AB/AY

Fig. 12 Shows one DGC and LGC which both make direct contact with the NL. AB/AY

3.1.4 DISTRIBUTION AND LOCALIZATION OF NEUROSECRETORY CELLS

A comprehensive map of DGC and LGC localized in the buccal ganglia has been constructed from serial sections and is seen in figure 13. From the LM observations, it seems clear that DGC and LGC are found in the fronto-dorsal, latero-dorsal and latero-lateral surface regions of the buccal ganglia (Figs. 4,8,10,11,13,14).

DGC have been detected in the ML and LL. They are not distributed all over the buccal ganglia. They are rather confined to the median and lateral lobes. DGC are mostly found in the arch of the LL, where LGC are also mostly observed (Fig. 4,10). In most sections, DGC have found in direct contact and seen to make a stratum adjacent to the NL as the first cell layer of the ganglion (Figs. 10,13).

DGC occur mostly as groups of four to nine cells (Figs. 10,13). They are occasionally found as single cells, either abutting to the NL (Fig. 9) or among other cells in the periphery of the ganglia (Fig. 7).

LGC are often found together with DGC in the same locations, the ML and the LL. However, they are mostly situated among other neurons in the periphery of the

ganglia, away from the NL (Fig.10). Like DGC, LGC are found either a group of cells which is up to four or single cells (Figs. 4,10,11).

Fig.13 Shows a group of DGC making a stratum adjacent to the NL in the arch of the LL. A few DGC are seen in the ML. AB/AY

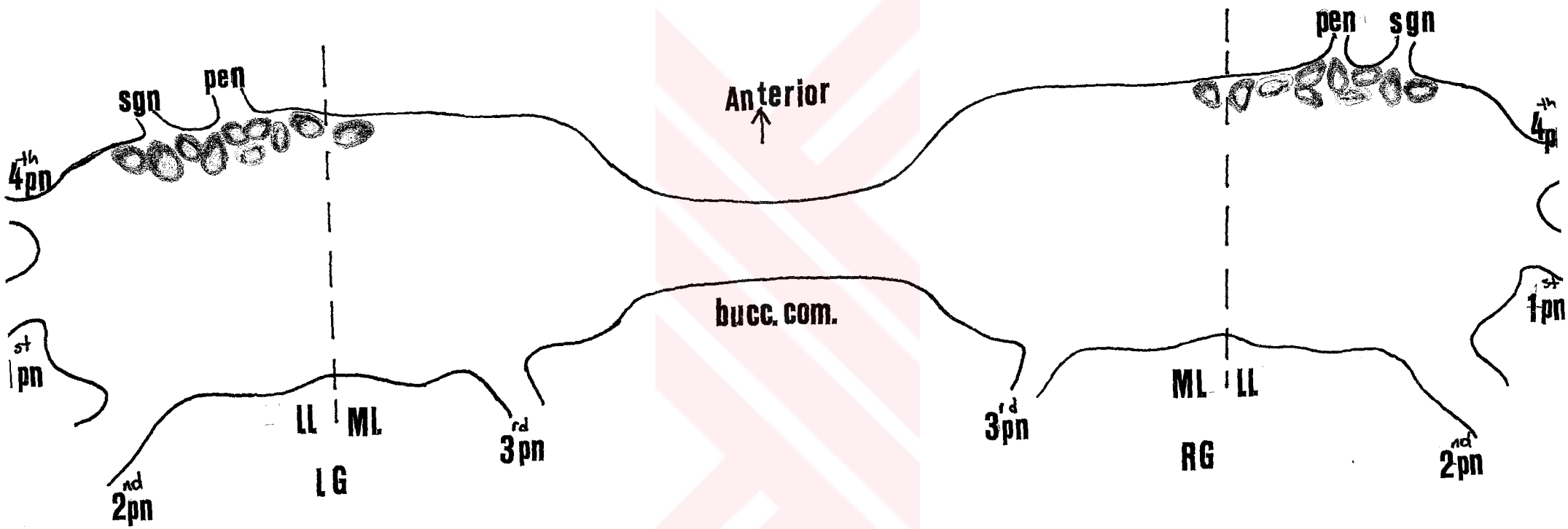


Fig. 14 Diagrammatic representation of the right and left buccal ganglia of H. pomatia showing locations and distribution of DGC and LGC identified with AB/AY histochemistry.

The localizations of DGC and LGC are followed in the serial sections (Fig. 11), and their localizations and positions in the ganglia are shown in a diagram (Fig.14). They are mainly localized in the latero-dorsal areas of each ganglion (Fig.10). That is to say, the number of DGC and LGC encountered in this region exceedes to that of those observed in other areas.

It is observed that the buc. com., cerebro-buc. con. and associated nerves of the ganglia do not contain LG and DG cell populations.

To tabulate the number of NSC studied (LGC and DGC), ten sets of ganglia were thoroughly examined by sectioning horizontal, transverse and longitudinal. The results are shown in Table I.

The total number of NSC in the buccal ganglia is given in table I. The number of NSC in each ganglion is about the same, with few exception. For example 2nd,5th and 6th blocks from the horizontal sections contain unequal number of NSC in the right and left ganglia. However, 1st, 8th, 9th and 10th blocks of the same plane of sections contain equal NSC in both ganglia. The number of NSC may differ from animal to animal ranging from 26 to 46 cells per ganglion.

Table 1. Reveals the total number of NSC distinguished in the right and left ganglia respectively in ten sets of blocks.

Cutting Plants of Blocks	1	2	3	4	5	6	7	8	9	10	To tal	Mean Number
Horizontal	14; 14	13; 23	15; 17	22; 17	21; 14	20; 13	15; 13	13; 13	14; 14	14; 15	314	31,4
Transverse	17; 18	21; 18	19; 18	21; 25	21; 18	18; 18	17; 19	21; 17	19; 20	21; 25	391	39,1
Longitudinal	23; 23	18; 19	19; 20	18; 21	22; 22	20; 19	21; 23	18; 21	19; 20	23; 22	412	41,2

The number of DGC and LGC was separately tabulated in each ganglion (Table 2). Horizontal sections were used for this tabulation, since the NSC were better observed due to the plane of the blocks. For the same reason, photomicrographs were taken from the horizontal sections. In the table 2, it is obvious that DGC outnumber LGC in the right and left ganglia. However, DGC and LGC are individually found more or less equal in number in both ganglia. The minimum and maximum numbers of DGC and LGC are in the range of 13 to 20 cells and 2 to 7 cells respectively.

3.2 ELECTRON MICROSCOPE OBSERVATION

In the EM studies, the NSC was firstly singled out in the epoxy section stained with toluidine blue, and then it was prepared for ultrastructural studies. Only one possible LGC stained lightly blue and situated centrally marked in figure six has been examined at the EM level in this work. Due to the limit of time, the ultrastructure of other NSC types has not been studied in detail.

3.2.1 ULTRASTRUCTURE OF NEUROSECRETORY CELLS

The NSC was readily recognized at the EM level by their inclusions in the cytoplasm (Fig. 15).

Table 2. Depictes the number of DGC and LGC in the Right and Left ganglia respectively in horizontal sections.

	Right Ganglion (RG)		Left Ganglion (LG)	
Blocks	DGC	LGC	DGC	LGC
1	13	4	13	3
2	13	3	14	7
3	13	3	14	2
4	16	3	17	3
5	16	5	20	5
Total	71	18	78	20
Mean Number	14,2	3,6	15,6	4

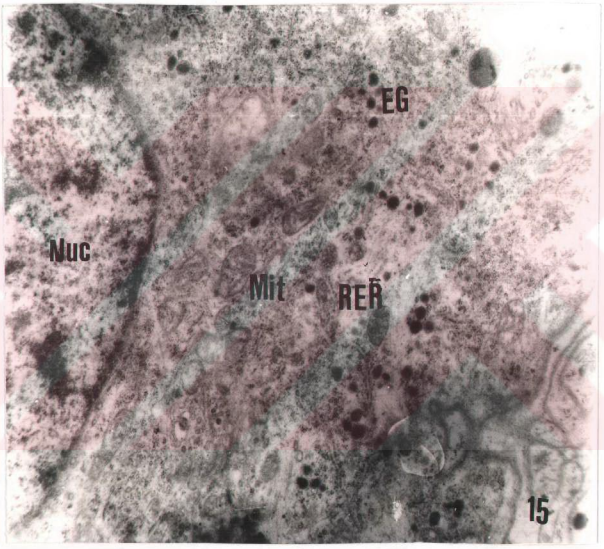


Fig.15 Electron micrograph of the NSC observed in Figure six. x 14700

The NSC examined typically containes membrane bound electron dense EG, but the cytoplasma is not over populated by secretory inclusions (Fig. 15). EG have a homogeneous electron-opaque dense core which completely fills the granule. The delimiting membrane of the EG appears to be smooth. The cytoplasm is mostly occupied by well developed RER. Mitochondria with electron dense matrix, Golgi bodies, are the other organelles conspicuosly seen in the cytoplasm. Moreover, the cytoplasm is heavily populated with ribosomes, some of which are attached to the endoplasmic reticulum. The nucleus occupies a central position with a distinct chromatin structures. The shape of nucleus which is ovoid (Fig. 15).

3.2.2 NEUROSECRETORY CELLS PROCESSES IN THE CONNECTIVE TISSUE

Close examinations with EM have showed that the connective tissue surrounding the ganglia has NSC processes or profiles which typically contain secretory inclusions with varying density and size (Figs. 16,17).

RER, Golgi apparatus and ribosomes are not present in NSC processes, but a few number of mitochondria smooth

endoplasmic reticulum and microtubules were observed (Fig. 17). None of the processes observed has been traced up to their cell bodies in serial sections. However, it is likely that they are fine cytoplasmic extensions, not axon endings. Each process contains electron-dense secretory granules with a mean diameter of 200 nm or 350 nm (Figs. 16,17).

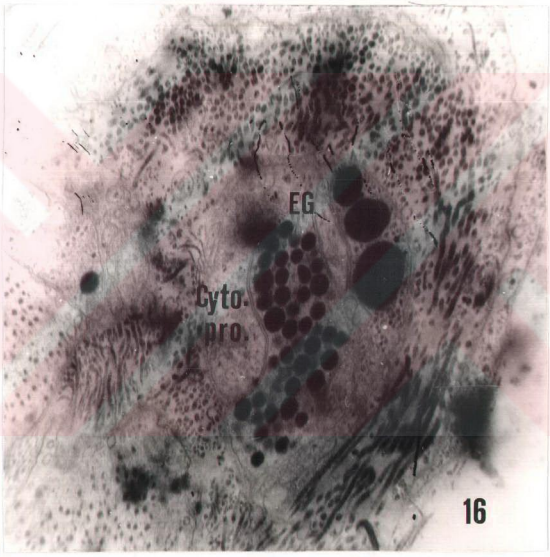


Fig. 16 Shows cell processes containing EG in the connective tissue. x 22000

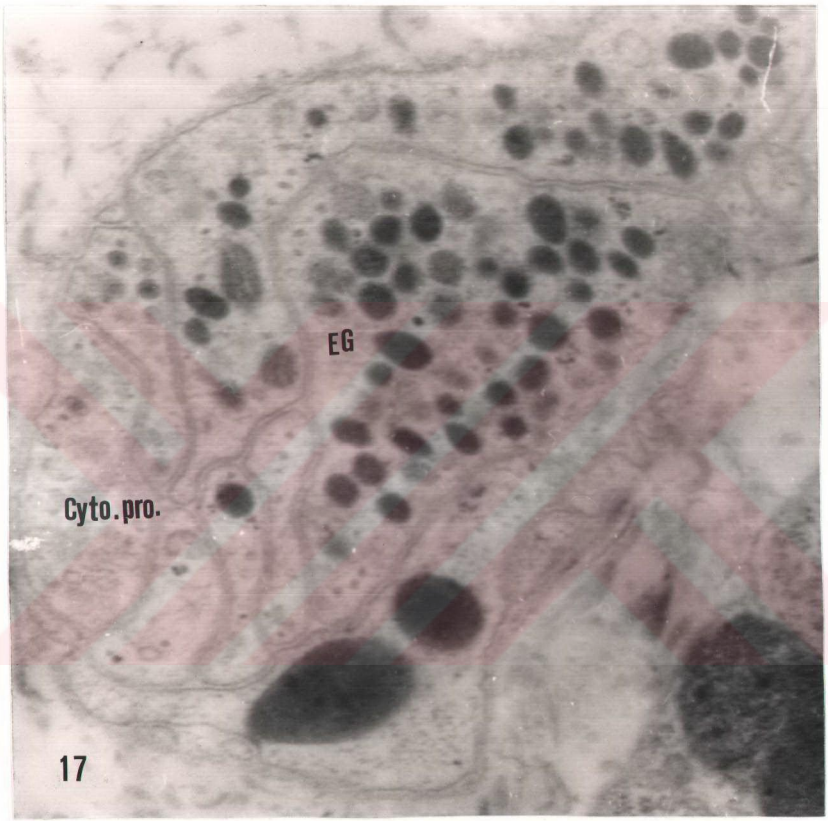


Fig. 17 Shows cell processes containing EG in the connective tissue. x 56000

CHAPTER IV

DISCUSSION

4.1 LIGHT MICROSCOPE OBSERVATION

In the identification of NSC by classical staining methods used in early studies, CH and PAF, are not adequate for detailed differentiation of NSC. With the classical methods, other substances such as pigments are also stained. For this reason, PAF is not considered as reliable diagnostic method for classification of NSC (Wijdenes et. al., 1980; Kai-Kai and Kerkut, 1979). It was reported that the AB/AY method is superimposed upon classical method on the distinction of NSC types.

With the AB/AY technique, it is possible to differentiate among PAF (+ve) and PAF (-ve) cells. The color differences in NSC obtained with this method are supposed to reflect different ratios of strong and weak acid groups in the secretory materials (Peute and Van de Kamer, 1967). A greater differentiation of NSC types was

achieved by the AB/AY method, depending on the chemical composition of their contents (Van Minnen et. al., 1977).

At the beginning of the present study, NSC were detected by the PAF method. These PAF (+ve) cells were further classified as DGC and LGC with the AB/AY method. In the rest of the study, the AB/AY method was prefered to PAF for the detection of NSC. In the present work, the nomenclature depending on the AB/AY method was preferred for use for these cell types. This nomenclature has also been chosen by other workers for classification of NSC in various Pulmonates (Wandelaar - Bonga, 1970; Boer, 1965; Wijdenes, et. al. 1980).

In sections stained with AB/AY method, NSC were observed in distinct green colors, dark green and light green. The coloration of these cells are interesting, since the cells reacted with the shade of green can be observed in the lighter tone of the same green color. That is to say, the kind of green color in perikarya did not change throughout the sections but the intensity of the color was graded. This tone difference in sections may be due to the quantity of neurosecretory material present in the perikarya.

Many authors have studied the NSC by the AB/AY method

in L. stagnalis. Seven types of cells were classified (Wandelaar - Bonga, 1970) and localization of these cells in the central nervous system have been reviewed in chapter one. In the cerebral ganglia of Melanpus bidentatus (Pulmonate, Basommatophora) the identified NSC by the AB/AY method have been also reviewed in the same chapter.

Wijdenes et. al., (1980) have classified NSC by the AB/AY method in the Central Nervous System of three Pulmonate Stylommatophora (D. reticulatum, H. aspersa and A. hortensis). They have distinguished BrGC, GC, YGC, LGC in the cerebral, parietal, pleural ganglia and single visceral ganglion.

Although NSC in the central nervous system of Pulmonates (Basommatophora and Stylommatophora) have been studied extensively by many authors, NSC of the buccal ganglia of these snails have attracted very little attention.

In Basommatophora, namely L. stagnalis and M. bidentatus NSC in the buccal ganglia have been studied extensively by Wandelaar - Bonga (1970) and more recently by Ridgway (1987). In Basommatophora, neither L.stagnalis (Wandelaar - Bonga, 1970) nor Bulinus truncatus do contain NSC, but M. bidentatus possesses NSC named as GC localized

in the dorsal part of the buccal ganglia (Ridgway, 1987).

In Stylommatophora, Wijdenes and his coworkers (1980) have reported that BrGC in H. aspera, DGC in D. reticulatum and A. hortensis are located in the dorsal part of the buccal ganglia. However, the exact localization of NSC reported in the dorsal region of the ganglia have not been well identified by these authors.

If the cell types observed in the buccal ganglia of Stylommatophora are compared, it is difficult to establish the equivalance of NSC described in the present study, with those found in H.aspersa, D.reticularis, A. hortensis (Wijdenes et. al., 1980). However, it may be possible that DGC of Helix pomatia and DGC of D.reticulatum and A. hortensis can be corresponded to some extend on the basis of the similarity found in staining affinity and general positions of the cells considered. However, we need more information on these cells, to make proper homology between them. It is also not possible to make homology between any of the NSC of the present study and GC of M. bidentatus (Basommatophora).

There is a great similarity between the central nervous system of H.aspersa and H.pomatia (Kunze, 1921). The general structure of the nervous tissue and

localization of the NSC are the same in both snails (Bayraktaroğlu, personal communication). Therefore, it seems possible that LGC and DGC described in the H.pomatia may also present in the buccal ganglia of the H.aspersa. With approximately similar localizations and positions. For this reason, BrGC reported by Wijdenes et. al. (1980) in H.aspersa can not be homology of neither DGC nor LGC of H. pomatia. It seems clear that the homology of NSC in the buccal ganglia of various Pulmonate species can not be suggested as homology of each other.

Observations on NSC in the buccal ganglia of Basommatophoran and Stylommatophoran snails and the finding of present work have been summarized in table 3.

In the nervous system of vertebrates and invertebrates there is a tendency for NSC to aggregate to form rather distinct cell groups. For example, the hypothalamic supraoptic and paraventricular nuclei of vertebrates, pars intercerebralis of insects, most NSC in L. stangnalis and the cerebral GC in Helix are found as cell groups in distinct areas of the nervous system.

Table 3. Summarizes NSC and their numbers in the buccal ganglia of Basommatophora and Stylommatophora snails.

STUDIES	Pulmonate: Stylommatophora				Basommatophora		
	H.pom.	H.asp.	D.ret.	A.hort.	B.trun	L.sta	M.Bid.
1					-	-	
2		BrGC (8-10)	DGC (8-10)	DGC (8-10)			
3							GC (3)
4	DGC & LGC (14 & 3)						

1 = Wandelaar - Bonga (1970)
2 = Wijdenes et. al. (1980)
3 = Ridgway. (1987)
4 = Present Work

H.pom. = Helix pomatia
H.asp. = Helix aspersa
D.ret. = D.reticulatum
A.hort. = A. hortensis
L.sta. = L. stagnails
B.tru = B.truncatus
M.Bid. = M. bidentatus

Similarly, observations made in this study have showed that LGC and DGC are situated in the certain areas of the buccal ganglia. They are confined to the LL and neighbouring part of the ML. The restricted localization of DGC and LGC does represent a pattern encountered throughout animal kingdom. The pattern exhibited by these cells is a typical feature of classical NSC which form clustured ganglionic nuclei. The distribution of NSC in the ganglia, in fact, is a rather typical case, since NSC form a cell layer between the NL and the neurons of the buccal ganglia. LGC have not been encountered in the ML. In other words, The ML contains only DGC which are very few in number in this lobe. However, the LL contains both DGC and LGC in groups of up to nine cells.

There are some similarities between DGC and LGC of H.pomatia. In general, they are similar in morphology, size, location and distribution in the buccal ganglia. DGC and LGC first detected in this study make direct contacts with the NL.

Although LGC and DGC observed in the central nervous system of Helix share the same staining affinity with LGC and DGC of the buccal ganglia, it is not clear whether LGC and DGC of these two ganglia constitute the same category of cells. Ultrastructural observations are required to

clarify this similarity between cell types.

In general, the number of NSC reported in the buccal ganglia of various Pulmonate species is less than that of other ganglia (Wijdenes et. al., 1980; Ridgway, 1987)(see table 3). This may be due to the size and function of the ganglia. The buccal ganglion of D.reticulatum, H.aspersa and A. hortensis contain 8-10 NSC and 1-3 NSC in M. bidentatus. However, the number of DGC and LGC H.pomatia is approximately 14 and 3 cells respectively. It is obvious that the buccal ganglia of Helix pomatia contain more cells than other Pulmonate species.

The NSC observed in the buccal ganglia of H.pomatia were not very much different from the ordinary neurons in their morphology. They may be round, spherical, elongated shape with a round spherical nucleus situated in the centre. The nucleus occupies most of the perikaryon leaving a very thin layer of cytoplasm. There has been no information about the morphology of NSC in other Pulmonates.

The total volume of the nucleus is bigger than that of the cytoplasm of DGC and LGC. It was reported that the significantly larger nucleus indicates high secretory activity, but the smaller ones indicate low secretory

activity (Ogrisegg, and Pohlhammer, 1985). In view of this knowledge, DGC and LGC of the buccal ganglia show high secretory activity.

NSC produce hormones which regulate the specific physiological functions of the animal. For example, in the cerebral ganglia, caudo-dorsal cells of L. stagnails produce ovulation hormone (Garaerts, and Bohlken 1976). LGC of the same snail produce a growth stimulating hormone (Joosse, 1975; Garaerts, and Bohlken 1976). There is no report available on the physiological functions of NSC in the buccal ganglia of Pulmonates. The functional significance of LGC and DGC described in this study has yet to be revealed in future studies.

LGC and DGC are closely associated with the NL without intervening any neuronal element observed under the LM. NSC occurring in direct contact with the NL have been reported in the central nervous system of Helix pomatıa and Helix aspersa (Whittle, 1978; Bayraktaroğlu, 1984). The term "synaptoid perikarya" was coined for these cells by Bayraktaroğlu et. al., (1988), because of their release sites constituted by the perikarya at the region of contact. LGC and DGC of the buccal ganglia resemble synaptoid perkarya in Helix pomatıa, in that, both type of cell perikarya are in direct contact with the NL. However,

type I cells of synaptoid perkarya have a widely scattered distribution throughout the nervous system and occur even along the connectives and nerves. Type I cells were encountered frequently and characteristically in shallow projection and elongated out pushings from the nervous system surrounded by the NL. However, type II cells of synaptoid perikaya were observed in a rather distinct area in the procerebrum of the cerebral ganglia. Type II cells possibly correspond to LGC lined up against the NL in the cerebral ganglia of Helix aspersa reported by Wijdenes et. al., (1980). For these reasons, LGC and DGC of the buccal ganglia and synaptoid perikarya in Helix do not represent the same cell types. However, it seems clear that they have at least one common property, that is the usual position of these cells adjetion to the NL. The close connection of these cells with the NL should suggest some indications of neurosecretory release phenomenon from the perikaya.

In many invertebrate and vertebrates release of neurosecretory materials takes place from axon terminals into the circulation in a specific organ called, neurohaemal organ, due to the blood-brain barrier (Maddrell and Nordmann, 1979). The existence of blood-brain barriers in many animal groups limit diffusion of materials rapidly into the circulation. Therefore, classical NSC follow the above pattern for release of neurochemicals.

Release of neurosecretory material from cell bodies has been suggested in early light and electron microscopical studies (Berlind, 1977). Kai-Kai and Kerkut (1979) have reported that the neurosecretory material in Helix aspersa may occur from four probable sites. According to these authors, one of the possible way is the release from the cell soma into the surrounding environment. The other sites for release are the connective tissue, some nerves such as medial lip, intestinal nerve and NP of the cerebral ganglia. In Helix aspersa , Whittle (1978) and Wijdenes et. al.,(1980) have also postulated separately somal release from NSC. However, none of these claims have been supported by EM studies.

Conclusive evidence for somal release has been provided by the demonstration of exocytotic release of secretory inclusions (EG and synaptoid vesicles) in synaptoid perikarya of Helix (Bayraktaroğlu, 1984, Bayraktaroğlu et. al., 1988, Bayraktaroğlu et. al., 1989).

In the present study, somal release for LGC and DGC of the buccal ganglia has been suggested on the basis of observations obtained by LM. It is most likely that the regions of contact between the perikarya and the NL constitute neurohaemal complexes. This suggestion is based not only to the position of cells identified,but also the

specific accumulation of stained secretory products in the perikarya abutting to the NL. However, conclusive evidence should be obtained for somal release of NSC described from EM studies.

It is known that molluscs have no blood-brain barrier (Maddrell and Nordmann, 1979). This is a marked contrast to the situation seen in many animals. Consequently, the NSC of molluscs do not need to release their neurohormones at definite neurohaemal organs. The release sites are widely distributed in this group of animal. In L. stagnalis, it is reported that the release of neurohormones occurs on the surface of nerves, commissures and connectives (Wandelaar Bongo, 1970, 1971; Boer and joosse, 1975). These places are called as neurohaemal areas. In Helix conversely, as already mentioned above, little is known about the location of the neurohaemal areas of the NSC (Whittle, 1978; Kai-Kai and Kerkut, 1979; Wijdenes et. al, 1980; Bayraktaroğlu et. al., 1988).

In view of these considerations and observations made in this study, somal release is an important phenomenon, by which a more rapid release of secretory materials occurs direct from the perikarya where the materials are synthesized. Therefore, the occurrence of somal release may partly explain why well-defined neurohaemal areas have not yet been reported in Helix .

4.2 ELECTRON MICROSCOPE OBSERVATION

One type of cells identified in Fig. 6 has been studied ultrastructurally. The cell contains EG with approximately 200 nm in diameter. EG do not appear to be evenly distributed and do not dominate the cytoplasm. EG have smooth limiting membranes around the dense cored secretory substance. EG in the cytoplasm indicate neuroendocrine status of the cell observed, since NSC typically contain EG(Scharrer, 1928; Bargmann, 1949). Therefore the cell observed is a possible NSC type.

It is very difficult to state whether the cell observed by EM is belong to LGC or DGC categories.

EM is a valuable tool in distinguishing NSC according to secretory inclusions. It may be possible that LGC and DGC can be categorized into subclasses by using EM in the future studies.

In addition, fine cytoplasmic processes penetrating into the NL were detected in the connective tissue under EM. Elementary granules in each profiles are various in morphology, size and appearance. It is possible that each profiles is an extension of different NSC.

Kai-Kai and Kerkut (1979) have also demonstrated projections of NSC in the connective tissue with LM and EM. They have suggested that the presence of dense clusters of granules in the connective tissue indicate release sites as one of the possible release sites in Helix aspersa.

The connective tissue in Helix are heavily vascularized (Pentreath and Cottrell, 1970). This area would be suitable for the release of materials into the circulatory system.

Observations obtained from the present EM studies on the cytoplasmic processes in Helix pomatia are in accordance with the findings of Kai-Kai and Kerkut (1979) in Helix aspersa.

In conclusion, there is no data on NSC in the buccal ganglia of Helix pomatia in the available literature. Thus, this study provides knowledge on neuroendocrine system of this ganglia. The histochemical evidence obtained in this work should be extended further by cytological and physiological methods.

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APPENDIX

Appendix A : Embedding and Staining Solutions

1 .The Epon Mixture (Luft, 1961)

Mixture A

Epon 812 ..... 5 ml

DDSA (dodecenyl succinic anhydride hardner) ..... 8 ml

Mixture B

Epon 812 ..... 8 ml

NMA ..... 7 ml

Final Embedding Mixture

Mix A .....13 ml

Mix B .....15 ml

DMP-30 (2,4,6-Tridimetylaminomethyl phenol) ..... 16 drops

2 .Aqueous saturated Uranyl Acelate solution :

Methanol ..... 80 ml

Distilled water ..... 20 ml

Uranyl acetate ..... 2 gr.

3 .Lead citrate

10 N NaOH ..... 0.5 ml

Bidistilled water ..... 50 ml

Lead citrate[$(C_6H_5O_7)_2Pb_3H_2O$] 200 mgr.

4 .Toluidine Blue

Distilled water	120 ml
Borax	1 gr.
Pronine	200 mgr.
Toluidine blue	800 mgr.