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THE MORPHOLOGY AND DEVELOPMENT OF NEUROSECRETORY CELLS IN THE BRAIN OF *Nereis virens* (ANNELIDA, POLYCHAETA)

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ABSTRACT

The brain of *Nereis virens* has been examined by light and electron microscopy, and one hindbrain nucleus, nucleus 20, has been studied in detail in animals of various ages. Four cell types, p, q, r, and s cells, have been found in this nucleus. The morphology of the p and r cells indicates that they may be functional neurosecretory cells from the time of their differentiation on, and especially in adult life. The structure of the developing nervous system in laboratory cultured larvae of this species has also been described. Nervous system growth parallels body growth, and occurs gradually, without metamorphic changes. Neurosecretory cells are among the last cells to differentiate in the larval brain, therefore it has been suggested that the brain has no endocrine function during larval life.

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MORPHOLOGIE ET DEVELOPPEMENT DE CELLULES NEUROSECRETRICES CHEZ *Nereis virens* (ANNELIDA, POLYCHAETA)

RESUME

Le cerveau de *Nereis virens* a été étudié à l'aide du microscope photonique et du microscope électronique. Le noyau 20 de la partie postérieure du cerveau a été examiné chez des animaux d'âges différents. Quatre types de cellules, les cellules p, q, r, et s, ont été trouvés à l'intérieur du ce noyau . La morphologie des cellules p et r indique que ces cellules ont une fonction neurosécrétrice à partir du moment où elles apparaîssent dans le cerveau, mais en particulier chez les adultes. La structure du système nerveux en développement des larves de cette espèce, élévées en laboratoire, a aussi été décrite. Tout comme la croissance du corps, la croissance du système nerveux se produit graduellement et sans métamorphose. Les cellules neurosécrétrices sont parmi les dernières cellules à se différencier dans le cerveau de la larve, c'est pourquoi il a été suggéré que le cerveau n'a aucune fonction endocrine durant le stage larvaire.

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TABLE OF CONTENTS

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PREFACE

The following thesis comprises a morphological study of the development of a particular "neurosecretory" cell group in the brain of the Annelid Nereis virens. This cell group, "nucleus 20", has been recognized in other species of Nereid by previous workers, but this study is the first to identify and characterize all the component cell types within the nucleus. This is also the first account of the development of the nervous system in a Nereid larva, including information on the differentiation of nucleus 20 as a neurosecretory centre. The results of this study have led to several new conclusions with respect to neurosecretion in the Nereidae.

The bulk of this research is reported in four manuscripts (Chapters 3,4 and 6, and Appendix II) which are to be submitted for review by scientific journals. Two introductory chapters and a single discussion have been added to the thesis in order to give some unity to the subject matter. The research which underlies this thesis, and the preparation of the thesis itself and the manuscripts which form part of it, are entirely the work of the candidate.

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While the inclusion of manuscripts co-authored by the Candidate and others is not prohibited for a test period, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is much more difficult in such cases.

CHAPTER 1

INTRODUCTION

NEUROSECRETION IN THE NEREIDAE AND THE NEREID LIFE CYCLE

THE NEREID LIFE CYCLE

Nereis virens (family Nereidae), the species under consideration in this study, is an errant polychaetous Annelid commonly occurring in salt water mud flats. These animals burrow extensively in the soft substrate and are presumably detritus feeders. The population that was studied here, a Deer Island, New Brunswick population, breeds during only one season of the year (April-May) and newly hatched larval forms appear in the mud at that time (Snow, 1972). Larval growth, as in other Nereids (Clark and Scully, 1964), involves an initial rapid addition of new segments, followed by a period of slower segment formation coincident with enlargement of existing segments. Regeneration of lost caudal segments is also possible. Maximum segment number, about 120-150 segments, is reached by the end of the second year of life. At this time metabolism becomes directed towards reproductive development. Somatic growth becomes limited to increases in segment size, and regeneration no longer occurs. Parenchymal cells appear in the coelom, and give rise to "gonadal clumps" of undifferentiated germ cell tissue. Over a period of a year or so these clumps proliferate either eggs or sperm (the sexes are separate in this species) which complete development in the coelom. The animals are ready to breed, at the earliest, when they reach four years of age (Snow, 1972; Snow and Marsden, 1974). A change in colour, from brown to green, occurs as mature gametes and coelomocytes build up in the body cavity, however, both sexes of this particular population breed in the atokous state, and do not undergo the sweeping somatic changes that are associated with epitoky in certain other Nereids (Clark, 1961). In the breeding season

swarms are observed, consisting entirely of ripe males. The females presumably shed their eggs in the mud without exiting from their burrows. These animals are assumed to be monotelic, with death of spent individuals occurring at the end of the breeding season (Snow and Marsden, 1974; Bass and Brafield, 1974). Although the life cycle of Nereis virens is among the longest reported for a Nereid (Snow and Marsden, 1974), in other features it is typical for this family (see, for example, description of Nereis diversicolor, Dales, 1950).

NEUROENDOCRINE CONTROL OF THE LIFE CYCLE

The Nereidae were the first polychaetes in which neuroendocrine activity was postulated. Scharrer (1936) suggested, on the basis of an histological study, that cells of secretory significance occurred in the brain (supraoesophageal ganglion) of Nereis virens. Subsequent experimentation (see below) has verified that in this family this ganglion is indeed the source of humoral regulators of the processes of growth, regeneration and reproduction.

Regeneration and Growth

Juveniles (as yet asexual individuals) which are normally still in the somatic growth phase of the life cycle, are capable of regenerating lost caudal segments. This regenerative capacity disappears if the brain is ablated (Casanova, 1955; Durchon, 1956a). The control is known to be hormonal rather than a direct nervous influence, since reimplantation of a juvenile brain into the coelom is sufficient to reinstitute regeneration of segments in a decerebrate host (Hauenschild, 1960). In Nereis diversicolor this brain hormone is said to be produced in increased amounts in direct response to segment loss,

and its secretion follows roughly a five-day time course. It reaches maximum quantity in the brain three days after segment removal, is released into the body during the fourth day and by the fifth day is again at a minimum in the brain (Clark and Ruston, 1963). Regenerative abilities decline gradually with age, and fully mature Nereids do not regenerate. It is presumed that the adult brain simply stops secreting the appropriate hormone(s), since implantation of juvenile brains into adults can facilitiate regeneration by the adult tissue, while the converse is not true (Scully, 1964; Clark and Ruston, 1963).

As well as influencing regenerative growth, the brain also affects normal growth. Segment addition ceases if the brain is removed from an intact growing juvenile, but can be induced to resume if the brain is reimplanted. In addition, adults which have already stopped growing can be made to proliferate some new segments by the implantation of juvenile brains (Clark and Scully, 1964).

Growth and regeneration appear to be very similar processes, both involving the formation of new segmental anlagen just anterior to the pygidium (Golding, 1967a; Olive, 1974). Ganglia from intact worms (ie. not undergoing regeneration) may still induce some regeneration in host animals deprived of their own brains (Olive, 1974), and regeneration will continue in a tail segment that is grafted to an intact host (Golding, 1967b). In addition, both functions decline in parallel with age, normally decreasing as sexual maturation begins (Clark and Scully, 1964; Clark and Ruston, 1963). It is therefore possible that both processes are controlled by a single cerebral hormone.

Reproduction

Reproductive changes in the Nereids include maturation of the gametes and bodily changes in preparation for spawning. These somatic changes may be slight, as in the case of Nereis diversicolor which reproduces atokally (Dales, 1950), or may involve full epitoky. In the latter event, quite radical alterations may occur in musculature, parapodia and setae (Clark, 1961). Both somatic and gametic maturation are controlled by secretions of the cerebral ganglion.

Epitoky

The existence of a factor in the immature animal, which is inhibitory to epitoky, was first shown as follows. Experimental epitoky was induced in worms which would not normally have metamorphosed for months, by ligation of the body (Durchon, 1948). The anterior segments of the operated animals proceeded to mature at a normal rate, while in the posterior segments, full epitokal transformation was precociously precipitated. The brain was localized as the source of this factor (Durchon, 1949, 1951, 1956), which has proven to be hormonal, since implantation of either an immature prostomium or brain, or injection of homogenate of the brain, are all effective means of preventing epitokal metamorphosis in worms having undergone brain ablations (Durchon, 1952, 1956b; Boilly-Marer, 1962; Hauenschild, 1956a). On the other hand, the brain of a sexually mature animal has no such preventive powers. Thus the epitoky-inhibiting factor, which has been called a "juvenile hormone",is thought to decline in quantity with aging, and by its absence to permit the onset of natural epitoky (Hauenschild, 1956b).

The same results have been noted in vitro. Epitokal modifications were observed in parapodia cultured in the absence of the brain, but were inhibited in cultures containing prostomia of

sexually immature worms (Durchon and Schaller, 1964; Malecha, 1967). Metamorphosis can never be induced in species that do not normally reproduce in the epitokal form (Durchon, 1956c).

Gametogenesis

In addition to inhibiting epitoky, the brain of immature Nereids also has hormonal control over gamete maturation. The effect was originally thought to be strictly inhibitory, since removal of the brain induced premature sperm development, as well as epitoky in the appropriate species. Replacement of the brain, either by implantation or injection of extract, forestalls this reaction (Durchon, 1951, 1956a,b; Hauenschild,1956a,b; Boilly-Marer, 1962). The same results have also been achieved in tissue culture experiments on isolated parapodia (Durchon and Schaller, 1963, 1964; Malecha, 1967).

However, examination of the progress of oocyte development in the absence of brain hormone has made it clear that the immature brain does more than simply inhibit gamete development. Varying results are observed when the brain is removed from a female Nereid, depending on the species and especially on the age of the operated worm. Normal oocyte formation follows a sigmoidal pattern. Three phases in growth can be identified:

a) a previtellogenic growth phase which is slow and involves little increase in oocyte diameter,

b) a vitellogenic growth phase which is more rapid and involves the formation of yolk-proteins and formidable increases in oocyte diameter, and

c) a post-vitellogenic growth phase, again slower and involving only minor increases in oocyte size, in which certain mucopolysaccharide components of the eggs are synthesized and final maturation occurs

(Bertout and Dhainaut, 1971). The actual sizes of the oocytes during any one of these phases, as well as the timing of development are species dependent. In general, previtellogenic oocytes would be about 25-50 microns in diameter, and would increase to 150-200 microns during vitellogenesis. Fully mature Nereid oocytes are of the order of 200 to 250 microns in diameter in most cases (Snow, 1972; Bertout and Dhainaut, 1971; Porchet, 1970; Schroeder, 1971).

If the brain is removed from females containing previtellogenic oocytes, these cells either remain refractory or degenerate. Brain ablation at a slightly later stage accelerates vitellogenesis, but these premature ova may not be viable if the brain is lost too early in this phase of development. During post-vitellogenesis brain ablation seems to have no effect at all on oocyte development (Hauenschild, 1966; Dhainaut and Porchet, 1967; Porchet, 1970; Schroeder, 1971; Golding, 1972). These results indicate that the brain has a trophic effect as well as an inhibitory one, on sexual maturation, and is required up to some critical stage in the life cycle for normal oocyte development to be assured.

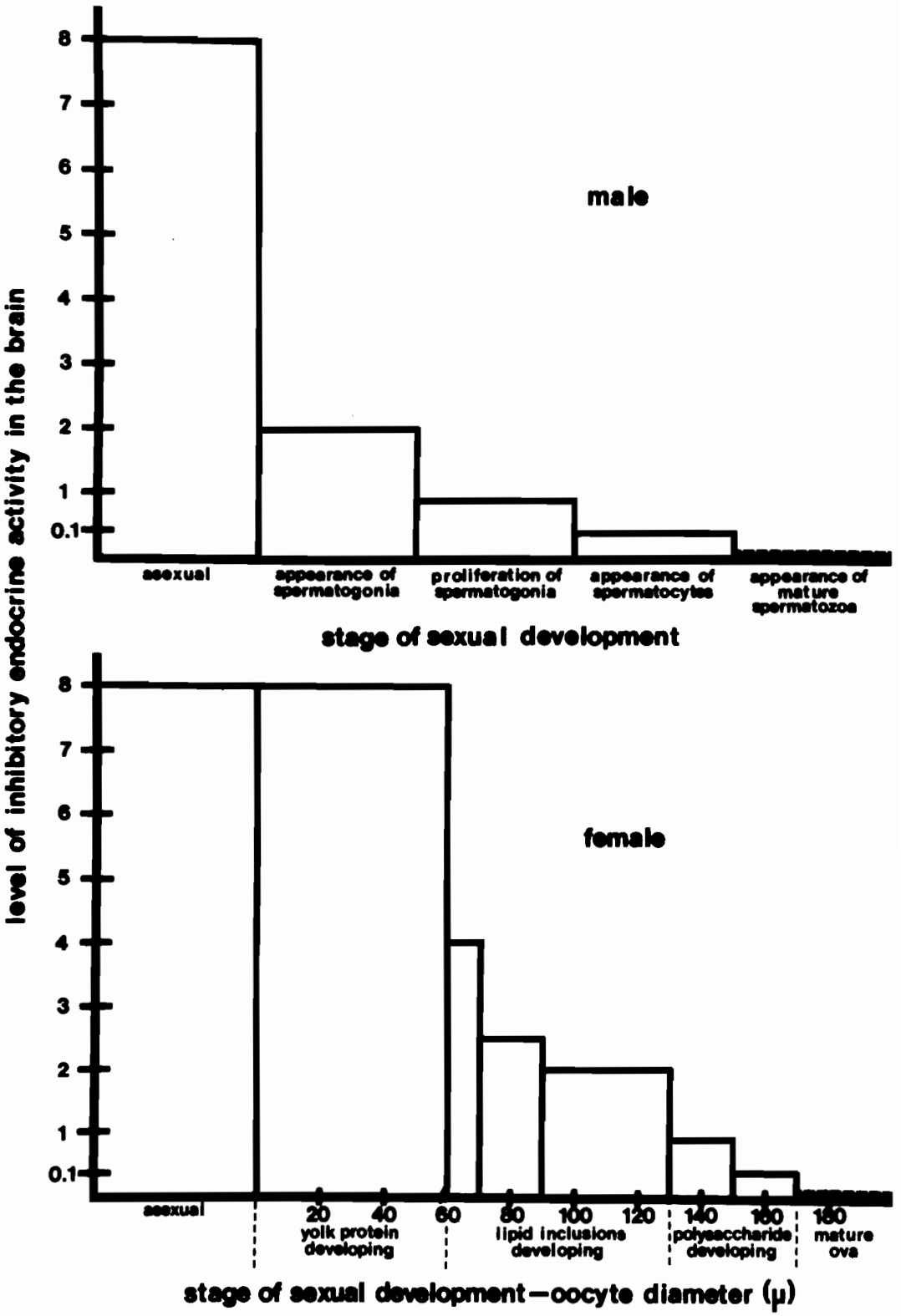
Reimplantation of a brain into the coelom of a decerebrate female permits maturation of the oocytes to be completed normally, but on a time course determined by the age of the donor brain. The younger the donor is, the longer the oocytes of the host will take to complete maturation (Hauenschild, 1956a, 1966; Hauenschild and Fischer, 1962; Durchon, 1962). It has thus been suggested that one cerebral hormone present in high titre in immature brains, and therefore inhibitory to sexual maturation, declines naturally with age, intermediate levels of the hormone being facilitatory to certain processes in gametogenesis (Hauenschild, 1956a, 1966). This hypothesis has been elegantly verified

in tissue culture experiments (Durchon and Porchet, 1970, 1971), in which an assay for the level of endocrine activity in the brain has been devised. Isolated male parapodia were cultured in the presence of extracts of either male or female brains of various ages. Levels of hormonal activity in the brain were determined according to how long spermatogenesis in the parapodia was inhibited. Although the actual levels of endocrine activity vary according to species and sex, a stepwise decline in the amount of gametogenesis-inhibiting factor in the brain occurs with age (Figure 1). Each decrease is sufficient to permit an advance in gametogenesis, although a certain quantity of the hormone apparently remains necessary for completely normal development of the germ cells, especially the oocytes.

Summary of Cerebral Endocrine Activity in Juveniles

In summary, it has been variously demonstrated that the supraoesophageal ganglion of Nereids exerts humoral control over growth/regeneration, epitoky and gametogenesis. It seems that decreased secretion of some positive factor with age, slows somatic growth and reduces regenerative abilities, and decreased secretion of an inhibitory factor permits the onset of reproductive development, including the processes of gametogenesis and epitoky. One and the same "juvenile hormone" may control both epitoky and gametogenesis, since the immature brain of an atokally reproducing species is capable of inhibiting precocious metamorphosis in decerebrate worms which would otherwise undergo epitoky (Hauenschild, 1956b; Boilly-Marer, 1962). It has also been suggested that the "juvenile hormone" involved in the suppression of sexual maturation, is the same hormone involved in the facilitation of growth and regeneration in immature animals (Hauenschild, 1966), since Platynereis dumerilii are seen to slow down their sexual

Figure 1. Decreases in the level of inhibitory cerebral endocrine activity with sexual development in male and female Perinereis cultrifera. Adapted from Durchon and Porchet (1971).


level of inhibitory endocrine activity in the brain
8
7
6
5
4
3
2
1
0.1
male
asexual
appearance of spermatogonia
proliferation of spermatogonia
appearance of spermatocytes
appearance of mature spermatozoa
stage of sexual development
female
asexual
20
40
60
80
100
120
140
160
180
yolk protein developing
lipid inclusions developing
polysaccharide developing
mature ova
stage of sexual development—oocyte diameter (μ)

development while regenerating. However, slower oocyte growth was not observed in regenerating specimens of Nereis diversicolor (Clark and Ruston, 1963). Thus although a single juvenile hormone theory is enticing in terms of economy, it cannot be considered proven. The actual number of hormones involved in the regulation of development remains unknown, and may turn out to be species dependent. The initial attempts which have been made at biochemical analysis of the brain of Nereis diversicolor, have so far only revealed one substance, of low molecular weight, with an inhibitory action on maturation (Cardon, 1970; Durchon, et al, 1963).

Regulators of Juvenile Cerebral Endocrine Activity

Both internal bodily and external environmental controls over the level of hormonal activity of the juvenile brain have been described. For example, early gametogenesis and epitoky was induced in both male and female Nereids by the injection of ripe gametes of the appropriate sex (Durchon, 1952; Porchet, 1967). It has thus been suggested that feedback from developing gametes may be instrumental in bringing about the natural decline with age in activity of the juvenile hormone(s), which permits the completion of gametogenesis. Also, a decrease in temperature was seen to cause a decrease in cerebral inhibitory endocrine activity (Durchon and Porchet, 1971), and a lengthened photoperiod has been reported to do the same (Hauenschild, 1955, 1960b, 1961). In the latter experiments, it was shown that the small light increases associated with the waxing of the moon were sufficient to trigger the final stages of sexual maturation in a given population of Platynereis dumerilii, with the net result being coordinated spawning by a large proportion of the adults about two weeks after full moon. This

rhythmicity will continue in the absence of light cycles, and can be entrained in the laboratory. The eyes are not necessary for this response, and Durchon (1971) has suggested that certain putative photoreceptor cells within the brain ("d" cells, Scharrer, 1936, 1937; or type IV cells, Dhainaut-Courtois, 1965) may be responsible instead.

Cerebral Endocrine Activity in Adults

Experimental evidence for adult hormones, as opposed to juvenile hormones (hormones in the immature brain), has been reported for only one Nereid to date. Müller (1973), noting an increase in the cystine metabolism of certain neurosecretory cells within the brain of Platynereis dumerilii females, correlated with the rapid vitellogenic phase of oocyte growth, has postulated the existence of a facilitatory "maturation hormone" in these worms.

ANATOMICAL CORRELATES OF HORMONAL ACTIVITY IN THE NEREID BRAIN

Secretory neurons

Although the first light microscope neuroanatomical description of the Nereid brain can be attributed to Hamaker (1898), the first author to suggest a secretory function for cells in the brain was Scharrer (1936, 1937). In all, she noted four cell types in the hindbrain of Nereis virens, Nereis diversicolor and Nereis pelagica which she believed to be neurosecretory.

In summary, she described the cells as follows: type a cells containing homogeneous cytoplasm, type b cells being spindle-shaped with vacuoles in the cytoplasm, type c cells being large rounded cells with vacuoles in the cytoplasm containing finely granular material, and type

d cells being blister-like, that is being primarily vacuolate. She suggested that d cells were the remnants of other cells (presumably c cells) which had secreted all their product. Scharrer did not, however, definitively localize these cell types within the hindbrain, leading to much confusion among subsequent authors. Possibly as a result, several other classifications of neurosecretory cells in the Nereids have arisen. Golding (1967) has reviewed these studies, and drawn analogies between them as far as it is possible to do so. The entire problem is compounded since several different species have been examined by the various authors.

The b cells have since been identified as sensory cells of the nuchal nucleus (nucleus 18)[1], (Whittle and Zahid, 1974), and the d cells have been described as possible photoreceptor elements (Dhainaut-Courtois, 1965), rather than secretory neurons. Also, there is still disagreement among authors as to just which neurons are actually the a and c cells (Golding, 1967; Dhainaut-Courtois, 1968a), thus Scharrer's classification would seem best relegated to historical status.

The most recent descriptions of the brain, which are based on ultrastructural as well as histological observations, appear to be more meaningful. Golding (1967b) has found cells containing neurosecretory-like dense granules in 22 of the 26 ganglionic nuclei of Nereis diversicolor. He attributes possible neurosecretory function to two of these nuclei, 20 (X1XS) and 22, because of the especial prominence of granules and synthesizing organelles in the cells of these particular

Footnote

[1]The ganglionic nuclei are numbered according to Holmgren (1916). For a pictorial representation of the Nereid brain, refer to Figure 1 in Chapter 2 of this thesis.

nuclei. Two cell types, distinguishable on the basis of granule size (average diameters 1000 A and 1250 A), were found in nucleus 20.

Dhainaut-Courtois (1966a, 1967, 1968a) has described six cell types in Nereis pelagica and Nereis diversicolor, and attributed neurosecretory function to two of these types which are strongly paraldehyde fuchsin-positive[1] and very rich in elementary granules. Type I cells, with granules ranging from 1333-1555 A, were found in nuclei 7 and 20. These cells reach maximum number and staining intensity with paraldehyde fuchsin in animals lacking reproductive products in the body, and have thus been suggested as a source of juvenile hormone(s). Type II neurons, which occur also in nucleus 20 and have an average granule diameter of 1666 A, reach maximal number in animals which contain developing gametes, and have no suggested function.

Unfortunately, there is no proof that any of these neurons secrete anything, since their processes have never been traced to their terminations, and their "products" have never been isolated. The qualification that they are only "possible" neurosecretory cells should be maintained until additional evidence as to their true nature becomes available. However, given the strong experimental evidence for secretion by the brain, and given the structural resemblance of these cells to other known neurosecretory cells (Bern, 1962), they would seem to deserve the consideration they have inspired.

Footnote

[1]It should be pointed out that, in general, Annelid neurosecretory cells seem to be PAF-positive even at times when they are actively secreting, in contrast to the situation in certain other neurosecretory systems, where PAF staining occurs only when product is being stored in the cells and is not being released (see Clark, 1965).

The infracerebral gland

In addition to secretory neurons located within the brain proper, an infracerebral gland, which is located on the ventral surface of the brain, on the outside of the connective tissue capsule, has been suggested as an hormone source. This gland is apparently derived from the peritoneal cells covering the capsule (Whittle and Golding, 1976; Dhainaut-Courtois, 1968b). In most Nereid species two cell types are recognizable in the gland, the C_1 (Dhainaut-Courtois, 1968b) or a cells (Baskin, 1974) being epithelial in nature (cuboidal or columnar) and the C_2 or b cells being packed with dense-cored elementary granules similar to those found in secretory neurons of the ganglion. A third cell type, C_3 cells, also containing elementary granules, is found in some species (Whittle and Golding, 1976). C_2 cells are maximal in number in young worms, while C_1 cells increase in number and hypertrophy with age. In view of the constant morphology of C_2 cells throughout life, and the hypertrophy of C_1 cells with age, the infracerebral gland components have been considered secretory (Whittle and Golding, 1976; Golding, et al, 1968; Dhainaut-Courtois, 1968b; Al-Sharook, et al, 1975). This point has been argued however, since cyclical changes in these cells, which might be indicative of actual secretion, have not been observed (Tombes and Dhainaut-Courtois, 1974), nor have direct neuronal connections with these cells been demonstrated conclusively (Baskin, 1974).

Despite question as to its indigenous endocrine activity, the infracerebral gland is generally accepted as a possible neurohaemal organ, or release site, for secretory products of the brain (Golding, et al, 1968). Numerous blind neuronal endings impinge on the inner surface

of the capsule of the brain, in the immediate vicinity of the infracerebral epithelium, and nowhere else. Four types of endings have been described ultrastructurally. Three of these (α, β and γ) contain typical neurosecretory granules (Dhainaut-Courtois, 1966b, 1967) distinguishable mainly by size. It has been speculated that the α and β endings correspond, respectively, to axons of neuronal types I and II (Dhainaut-Courtois, 1967, 1968a,b), although the granules in the cell bodies are not identical to those in the terminals, and continuity of the cells with the endings has never been shown.

The fourth type of ending in this region is rich in large mitochondria, which often contain characteristic dense inclusions, rather than in neurosecretory granules. These so-called "secretory end-feet" have been traced to their cells of origin in ganglionic nucleus 13, and have also been thought to have neurosecretory function (Golding and Whittle, 1974).

Although morphological data for neurosecretory centres in the brain are plentiful, for the most part it remains impossible to attribute specific functions to the variety of components which have been described. More questions seem to have been raised than answered, since, in addition to cells such as type I neurons which might conceivably produce a juvenile hormone (Dhainaut-Courtois, 1968a), other components persist beyond the time at which juvenile hormone production would be expected to stop. For example C_1 cells of the infracerebral gland hypertrophy with age, and the secretory end-feet of the nucleus 13 neurons persist unaltered throughout life (Al-Sharook, et al, 1975). In addition, Dhainaut-Courtois (1966a, 1968a) has noted that the type II neurons reach maximum numbers in middle-aged animals which have begun to manufacture genital products.

Morphological evidence would therefore seem to support the conjecture that a variety of hitherto undescribed neurohormones may exist in this family, rather than only one juvenile hormone.

THE PRESENT STUDY

Nereis virens has not to date been experimentally examined for neuroendocrine activity. However, it was noted in the course of other morphological investigations done in this laboratory, that N. virens is histologically very similar to other Nereids which have been well studied in this regard (Marsden, 1978; White and Marsden, 1978; Easdown, et al, in preparation). Also, as previously mentioned, its life cycle is not notably different from that of other animals in this group (Snow, 1972; Snow and Marsden, 1974). It is therefore presumed that Nereis virens may have neurosecretory control mechanisms analogous to those shown to exist in other members of its family.

In this study Nereis virens has been examined over the course of its life cycle, from larval stages through to sexual maturity, in an attempt to describe the location, morphology and development of putative neurosecretory cells within the brain, and to correlate any noted changes in these cells with known events in the life history, as well as with presumed endocrinological events.

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CHAPTER 2

GENERAL ANATOMY OF THE BRAIN (SUPRAOESOPHAGEAL GANGLION) OF *Nereis virens*

INTRODUCTION

A classical account of the arrangement of ganglionic nuclei within the brain of Nereis diversicolor (Holmgren, 1916) has not only proven useful to more recent investigators of the same species (ie. Golding, 1967), but has been found to be applicable to other Nereids as well (for example, N. pelagica, Dhainaut-Courtois, 1964, 1965, 1966a, 1968). In the course of the present study, Holmgren's description was found to be suitable for Nereis virens also, therefore his designations for the neuronal nuclei will be used throughout this thesis. His numbering system is pictorially reviewed in the following general anatomical descriptions of the brain of N. virens, which is provided as a reference for subsequent discussion of specific cerebral nuclei.

MATERIALS AND METHODS

Nereis virens in a range of sizes were obtained from Marine Research Associates, Ltd., Deer Island, New Brunswick. Whole prostomia were fixed 24 hours in Bouin's fluid, and paraffin embedded. Five micron sections were stained with either hematoxylin-eosin or the fluorescent Gomori-Bargmann chrome alum hematoxylin-phloxine method for fibrous glia (Zimmerman, 1967; Baskin, 1971). Phloxine stained material was viewed on a Leitz Orthoplan microscope equipped with a Ploempak 2 vertical illuminating fluorescence system. The light source was an HBO 100 mercury vapour lamp, and a standard H2 filter block was used to provide an excitation wavelength suitable for phloxine B (390-490 nm).

RESULTS

General Anatomy and Glial Morphology

The supraoesophageal ganglion (brain) of Nereis virens lies roughly within the quadrangle formed by the four eyes (Fig. 1), and is loosely attached by its nerves and connective tissue capsule to the dorsal epidermis of the prostomium. It is bathed by the coelomic fluid, and its ventral surface is in close apposition to the pulsatile dorsal blood vessel which serves the animal as a heart (Fig. 4E). An infracerebral gland, such as has been described in other Nereids (Bobin and Durchon, 1952; Dhainaut-Courtois, 1966b; Golding and Whittle, 1974), also exists in Nereis virens (Figs. 4E,5I).

Observations confirm that the brain is in general anatomy similar to the ventral nerve cord (Baskin, 1971). The neuropil is centrally situated, and the neurons surround it. Two distinct layers of glia envelope the neural components. An "inner cortex", which is quite heavily fluorescent in phloxine stained slides, covers the neurons and neuropil. An "outer cortex", containing relatively few fluorescing fibres, lies between the inner cortex and the collagenous capsule that ensheathes the whole brain (Fig. 2A,B). The glia push in among the neurons and are responsible for their separation into recognizable nuclei. In small worms the glial layers are less well developed, and the individual ganglionic nuclei are consequently harder to distinguish (Fig. 2C,D).

Although glial cytoplasm and cell boundaries are poorly defined by the light microscope, three types of glial nuclei were identified in hematoxylin-eosin stained material. The first type is oval, 6-8 microns in length, and is very pale staining, with only a thin layer of heterochromatin underlying the nuclear membrane (Fig. 3A). These nuclei

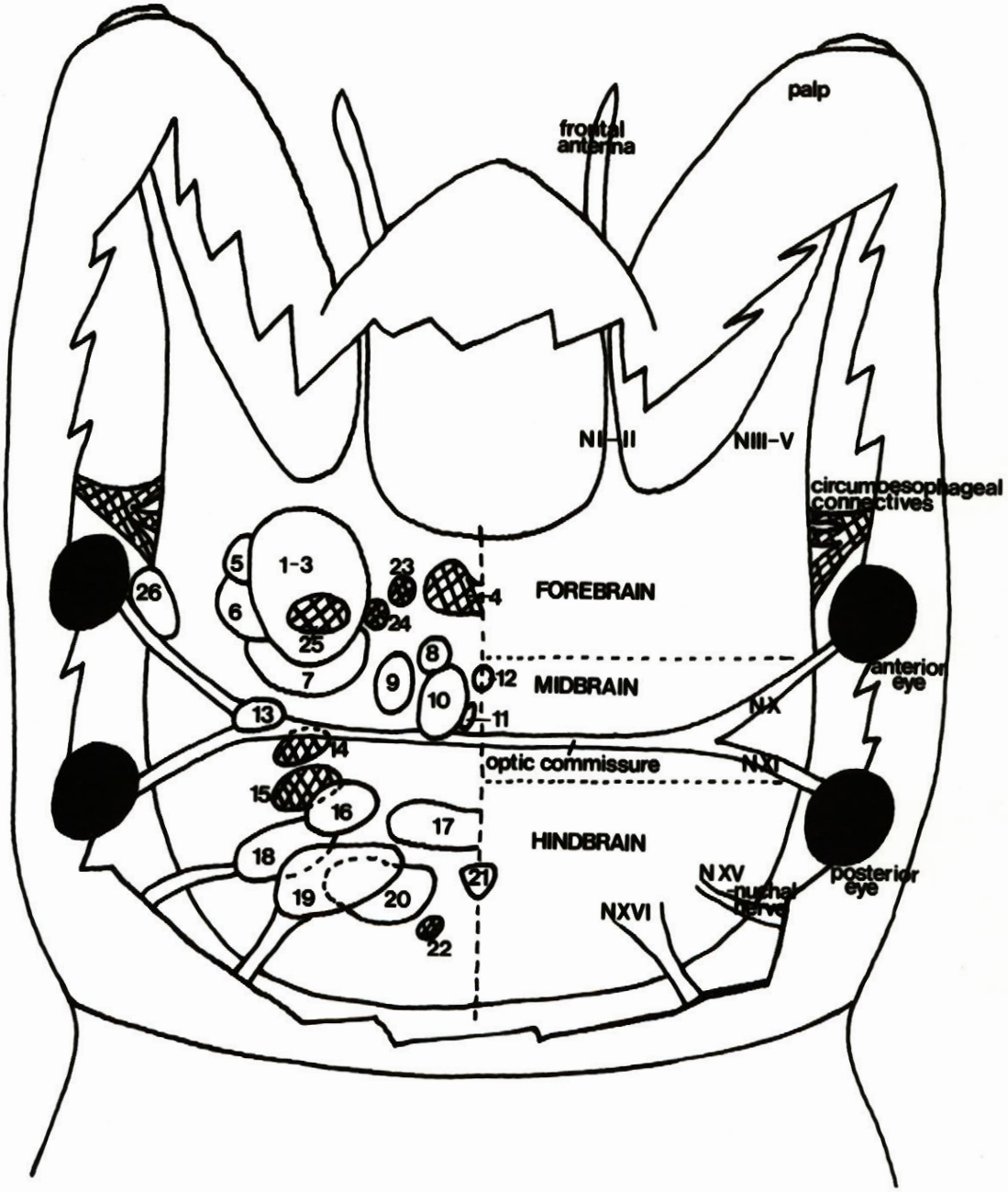
are found only in the outer cortex. The second type of nucleus is of similar size and shape, but stains more densely (Fig. 3B,C). It occurs in the outer cortex, especially right beneath the capsule, and also in the inner cortex. The third nuclear type, 4 to 5 microns long, is the darkest staining and is often seen surrounded by an obvious "spindle" of cytoplasm. This type of glia is found throughout the brain, but is most common in the neuropil and nerves, where it is usually oriented parallel to the adjacent neuronal processes, and as a "satellite" cell around individual neuronal cell bodies (Fig. 3A,B,C). A fourth possible glial type exists, a so-called "granulocyte" (Baskin, 1971). These cells are full of large brownish pigment granules which obscure the nucleus and prevent its description (Fig. 3D).

Neuronal Nuclei and Nerves

The locations of the 26 neuronal nuclei and of the major nerves of the N. virens brain, are depicted schematically in Figures 1 and 4, and are also shown in photomicrographs of actual sections through the ganglion (Fig. 5). It is convenient to refer to anterior, middle, and posterior portions of the brain, as indicated in Figure 1. The forebrain is defined as being that part of the ganglion which lies level with, or in front of, the anterior pair of eyes. Forebrain nuclei include the corpora pedunculata (nuclei 1-3), and nuclei 4 through 6, and 23 through 26 (Figs. 1; 4A; 5A,B). The midbrain, occupying the area between the two pairs of eyes, includes nuclei 7 through 14 (Figs. 1; 4B,C,D; 5C,D,E,F). The hindbrain, which is roughly level with the posterior eyes, is made up of nuclei 15 through 22 (Figs. 1; 4E,F; 5G,H,I).

The above observations confirm that the brain of Nereis virens is very similar in organization to that of certain closely related Nereids, especially Nereis diversicolor and Nereis pelagica, which have been previously studied (Holmgren, 1916; Dhainaut-Courtois, 1964, 1966a, 1968; Golding, 1967).

Figure 1. Dorsal view of the Nereis virens prostomium, showing the major nerves and the 26 ganglionic nuclei of the brain. Redrawn from Holmgren (1916). All nuclei are paired, except numbers 12 and 21. Nerves are designated in Roman numerals, nuclei in Arabic numerals. Ventral nerves and nuclei are cross-hatched.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FOREBRAIN
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12
11
MIDBRAIN
7
13
NX
anterior eye
14
optic commissure
NXI
15
16
17
HINDBRAIN
18
19
20
21
22
NXV
nuchal nerve
NXVI
posterior eye

Figure 2. Cross sections of N. virens brains stained with chrome alum hematoxylin-phloxine. Figures A and C are taken from adult and juvenile brains, respectively. Figures B and D are the same two sections viewed by fluorescence microscopy. The ganglionic nuclei are numbered and the glial layers marked with arrows. Outer cortex-oc, inner cortex-ic, neuropil-np.

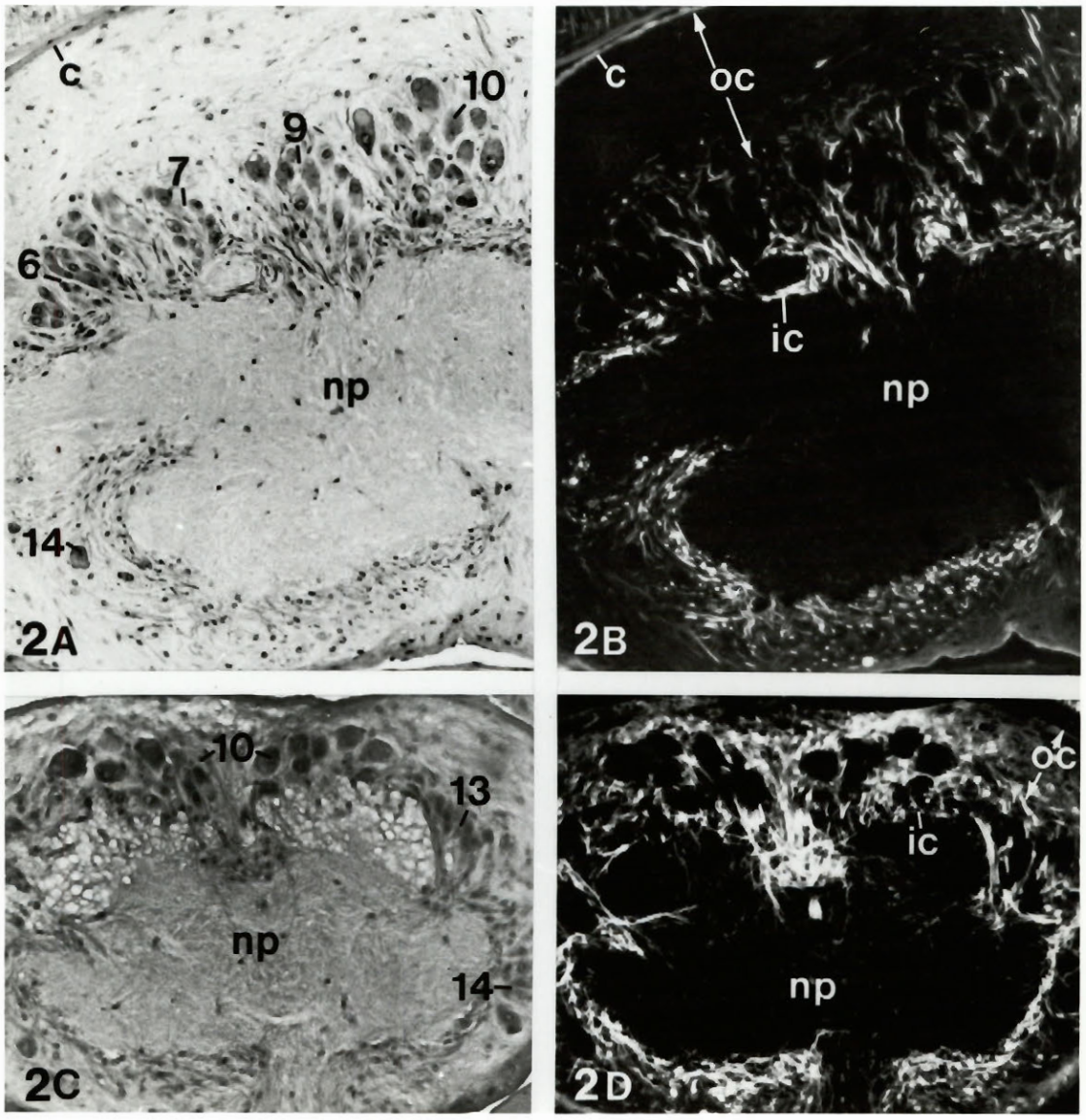
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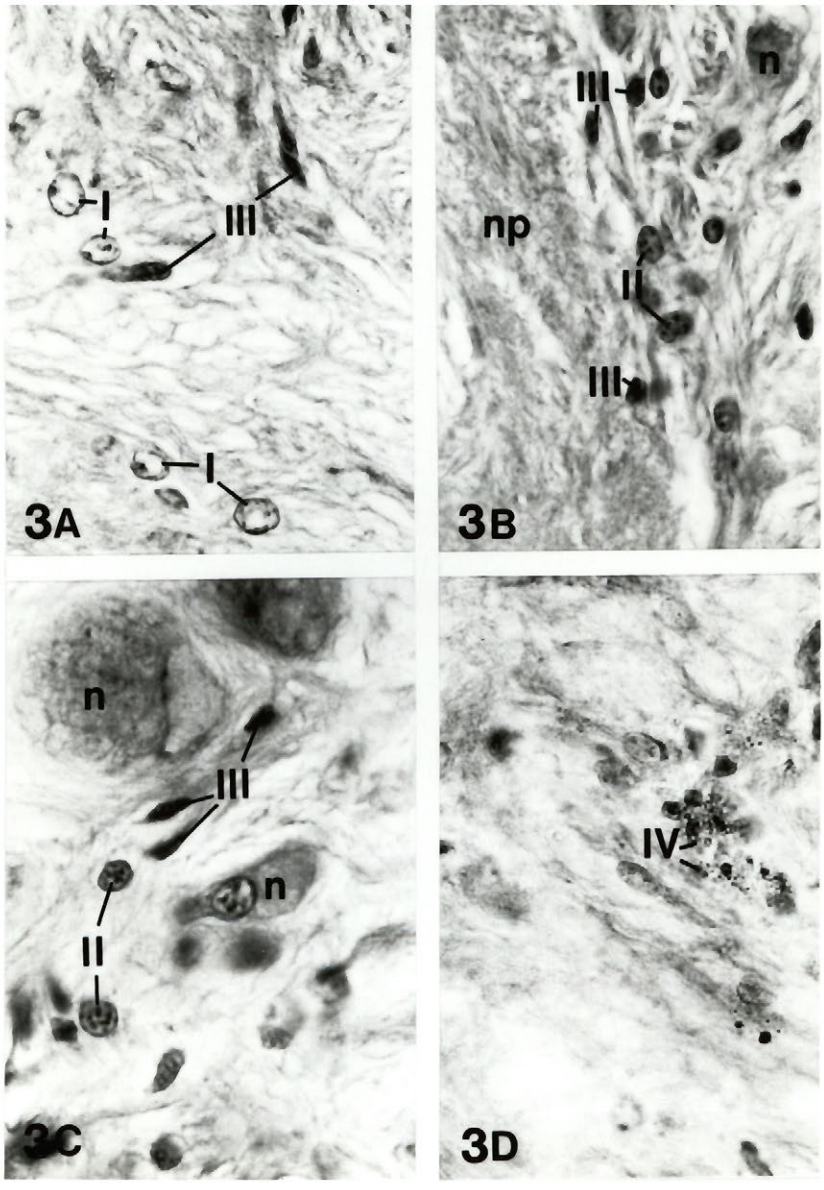
Figure 3. Hematoxylin-eosin stained sections through the brain, showing the four glial types which can be recognized at the light microscope level. See text for description. Neuropil-np, neuron-n.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II
3C
IV
3D

Figure 4. Schematic representations of cross sections through the brain, showing the positions of the 26 ganglionic nuclei, and the major nerves. Based on serial sections of the brain. Compare with micrographs in Figure 5. Nerves are designated in Roman numerals, nuclei in Arabic numerals. Neuropil regions are cross-hatched.

4A. Sketch of the anterior region of the brain.

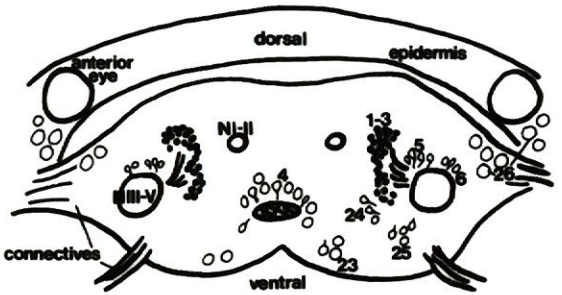
4B. Section at the forebrain-midbrain junction.

4C. Section through anterior portion of the midbrain.

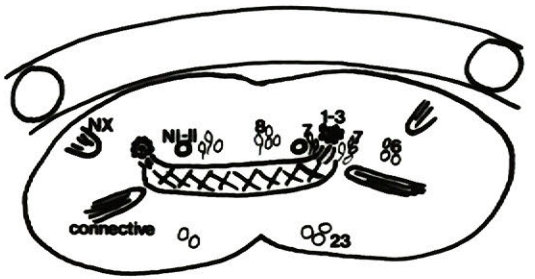
4D. Section through the posterior portion of the midbrain.

4E. Representation of the anterior portion of the hindbrain.

4F. Representation of the most posterior cells in the brain.



4A



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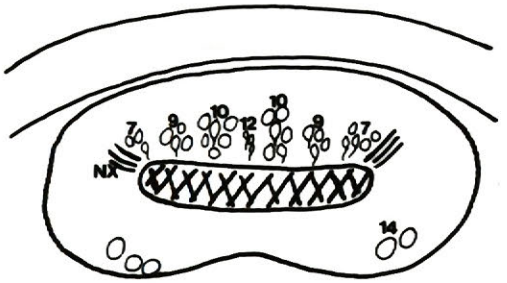

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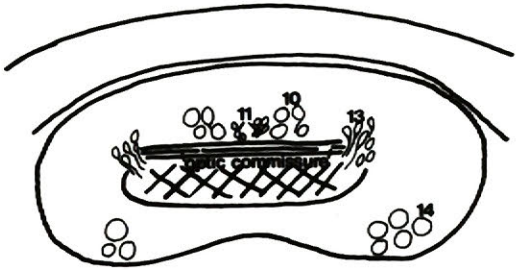
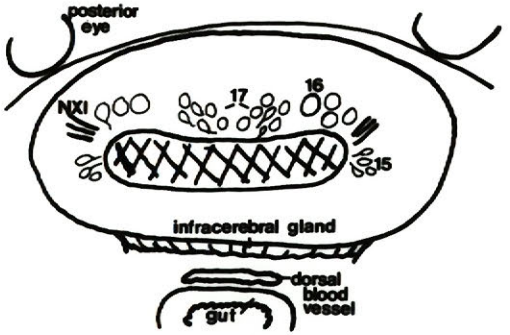
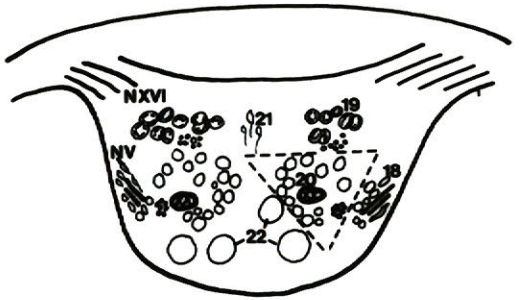


4D

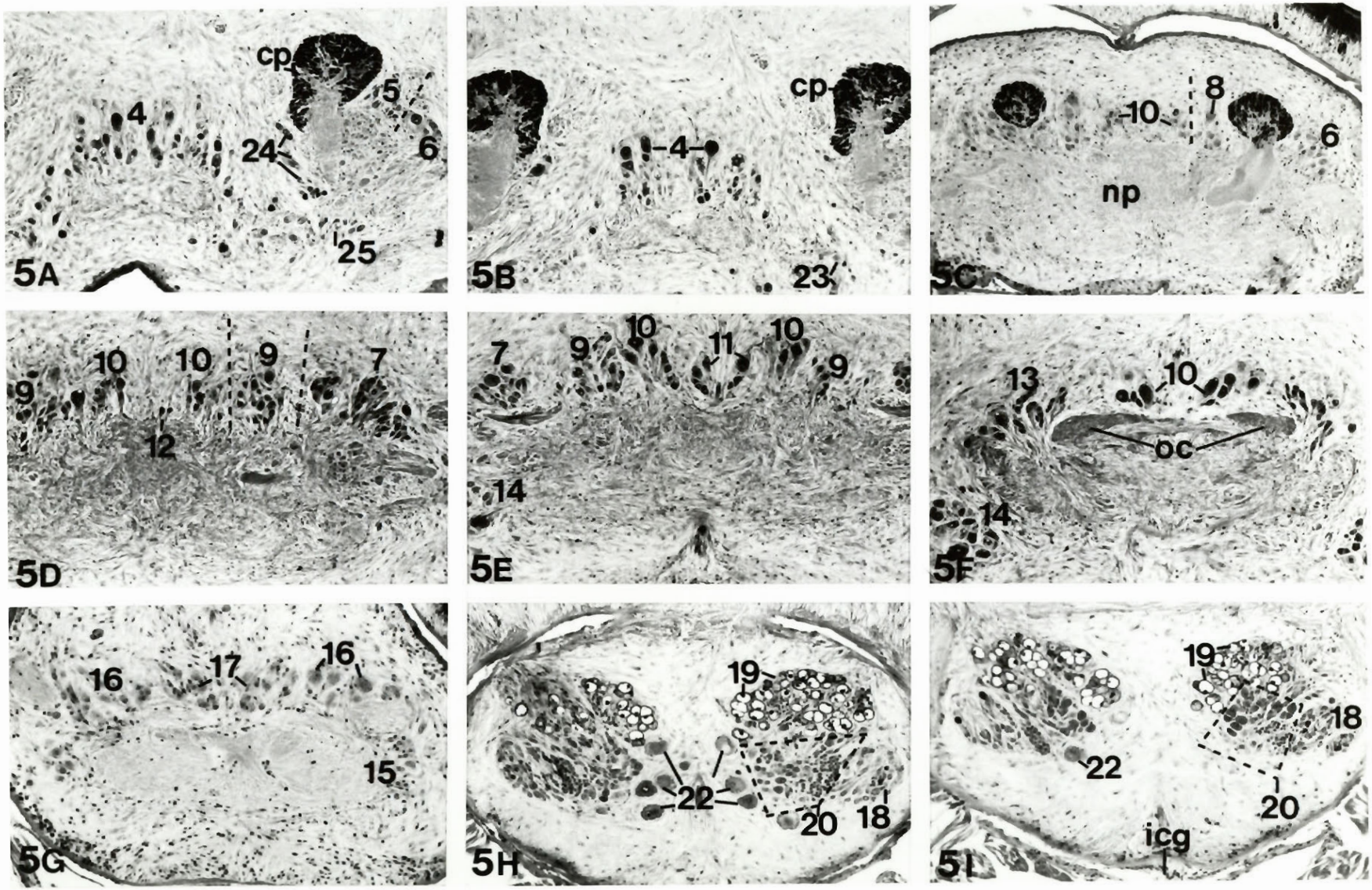


4E



4F

Figure 5. Light micrographs of hematoxylin-eosin stained cross sections through the N. virens brain, arranged in a rostro-caudal order. Ganglionic nuclei are designated in Arabic numerals, nerves in Roman numerals. Corpora pedunculata-cp, neuropil-np, optic commissure-oc, infracerebral gland-icg.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CHAPTER 3

AGE-RELATED HISTOLOGICAL CHANGES IN NEUROSECRETORY CELLS IN THE SUPRAOESOPHAGEAL GANGLION OF *Nereis virens* (ANNELIDA, POLYCHAETA)

ABSTRACT

The supraoesophageal ganglion of Nereis virens has been surveyed by light microscopy to determine the locations of probable neurosecretory cells. Neurons reacting with paraldehyde fuchsin are found scattered throughout the brain, but the majority are accumulated posteriorly in nucleus 20. This nucleus was examined in some detail, and the histology of four cell types is described. Two types (p and r) are strongly PAF-positive, and may be secretory. On the basis of other staining characteristics, and uptake of labelled cystine, it is concluded that p cells are rich in cystine and/or cysteine. A comparison of juvenile and adult brains revealed that the same cell types exist at both developmental stages, but that p and r cells increase in number and staining intensity with age. These findings are not consistent with the notion that these particular cells are the source of a "juvenile hormone" which has been reported to exist in Nereids. Rather it is suggested that the described cells are producers of a maturation and/or spawning hormone.

INTRODUCTION

Abundant evidence from brain extirpation and transplantation experiments, as well as tissue culture work, has made it clear that the supraoesophageal ganglion plays an endocrinological role in the life cycle of many Nereids. (For reviews see Baskin, 1976; Clark, 1965; Golding, 1974.) Briefly, it appears that the brain of the immature worm produces some sort of hormone which facilitates growth and regeneration of lost segments. The same, or possibly a second "juvenile hormone", exerts a simultaneous inhibitory effect on sexual maturation. Brain removal in an immature worm is generally followed by cessation of growth, loss of regenerative capacities, and the onset of processes normally associated with maturation. Thus it has been postulated that a natural decline in titre of the juvenile hormone(s) occurs with aging, allowing normal maturation to take place. Low levels of the hormone(s) are thought to be maintained, however, since they appear to be necessary for the completion of vitellogenesis.

The search for neurons in the brain which might be responsible for secreting the juvenile hormone(s) has not been extensive. A number of studies (reviewed by Golding and Whittle, 1977) indicate that there are indeed neurosecretory cells in the Nereid brain, but little attempt has been made to correlate the appearance of these cells with events of possible significance in the life history of the animals investigated. Clark and Bonney (1960), using paraldehyde fuchsin (PAF) staining as an indicator, found increased neurosecretory activity in certain supraoesophageal nuclei of Nereis diversicolor during tail regeneration. Marsden (1978) reported higher phosphotidylinositol

labelling in immature than in mature Nereis virens, in particular in the PAF-positive cells of nucleus 20 (X1XS). This latter nucleus comprises a large proportion of the PAF-positive cells in the Nereid brain, and has been presumed to be secretory on the basis of both light microscopic and ultrastructural studies (Scharrer, 1937; Dhainaut-Courtois, 1966, 1968; Golding, 1967). However, as Golding (1967) has pointed out, it is clearly not an homogeneous nucleus, and it has not to date been described in its entirety, although some of the cells within it have been characterized (Scharrer, 1937; Dhainaut-Courtois, 1966, 1968; Golding, 1967).

In the present study, specimens of Nereis virens were examined by light microscopic methods, including PAF staining, in order to clarify the cellular composition of nucleus 20, to define the locations of possible neurosecretory cells in the brain, and in particular to detail any changes that might be associated with aging and maturation in this species.

MATERIALS AND METHODS

Spawning adults were collected during spring breeding seasons (1977, 1978) at Deer Island, New Brunswick. Non-mature animals, of all sizes, were obtained throughout the year from Marine Research Associates Ltd., Deer Island. Worms were categorized according to weight and degree of sexual maturity (Table 1). The latter was judged by the appearance of coelomic fluid smears taken from animals representative of each group. A drop of coelomic fluid was spread on a slide, air dried, then fixed in 95% ethanol, and stained with hematoxylin and eosin. Smears were examined for the presence of reproductive cells (gametes or

gonadal clumps) and/or two types of non-reproductive coelomocytes as described by Snow (1972) and Snow and Marsden (1974).

Prostomia from animals of all sizes were fixed in Bouin's fluid, embedded in paraffin and sectioned at five microns. Slides were stained with paraldehyde fuchsin (PAF) according to either of two methods (Cameron and Steele, 1959; Meola, 1970), or with hematoxylin-eosin, or with a thiosulphation technique for the demonstration of cystine and/or cysteine (Castino and Bussolati, 1974). Victoria blue, as prepared by Dogra and Tandan (1964), was substituted for Alcian blue in the latter procedure (see Appendix II, this thesis). A second set of prostomia fixed in 5% formaldehyde in sea water was processed as above but stained with a performic acid-Victoria blue technique, likewise for the demonstration of cystine/cysteine residues (Dogra and Tandan, 1964). In all cases brains of various sizes were stained concurrently.

Cell counts were made of ganglionic nucleus 20 in selected brains. The total number of neurons in the nucleus was determined from complete sets of serial sections. Every fourth section was counted, and a correction for over-counting was made, using Konigsmark's (1970) split-cell correction formula.

Non-mature animals of all available sizes were labelled concurrently with ^{35}S-cystine (S.A. = 45 Ci/mM, New England Nuclear) by incubating them in sea water containing about .0002mg/ml (0.5 μCi/ml) of the precursor. Worms from each size group were sacrificed after 1,2,4,7 and 10 hours, and 1 and 2 days incubation. Their prostomia were fixed in Bouin's fluid, embedded in paraffin and sectioned as above. Slides were prestained with Delafield's hematoxylin and eosin, then coated with Kodak NTB2 emulsion. All radioautographs were processed

simultaneously and were developed in D170 (6 minutes) and fixed in 24% sodium thiosulphate (3 minutes) according to the method of Kopriwa and Leblond (1962).

RESULTS

Worms were divided into four groups according to the criteria detailed in Table 1. It should be emphasized that the life cycle, growth rate, etc., form a continuum, and that the animals were grouped developmentally only to facilitate data organization.

Brains of mature and maturing adults are qualitatively similar. Examination of PAF stained material reveals scattered PAF-positive cells throughout the brain. Nuclei 4-10, 13-17 and 22-25 each contain low numbers of such cells. (Nuclei numbered according to Holmgren, 1916.) However, about 60% of all the PAF-positive cells present in any brain are located in the hindbrain, in nucleus 20. This nucleus was therefore examined in detail, with the result that four cell types were recognized. The remaining nuclei did not contain PAF-reactive neurons.

Two types of PAF-positive cells exist in nucleus 20. Type "r" cells are identifiable in hematoxylin-eosin preparations as well as in PAF treated material (Fig. 1). These cells are large and pyriform, and are clumped at the most posterior margin of the nucleus, with processes running anteriorly into the central neuropil. Type "p" cells are bipolar, and lie laterally between the r cells and the cells of the nuchal nucleus 18 (Fig. 1). They appear to send processes to the dorsal epidermal nerve (nerve XIV, Holmgren, 1916). With the two PAF methods utilized here, differential staining results were obtained. Only the p cells stain by the Meola method. The Cameron and Steele method stains both the p and r cells, but the p cells are distinguishable due to their somewhat darker colouration (Fig. 1). In addition, p cells are the only

Table 1. Division of the animals examined into four groups on the basis size and sexual maturity.

GROUP	AVERAGE WEIGHT	COELOMIC CONTENTS*
mature (gravid) adults	7-10 grams	ripe eggs (200-275μ) or sperm gonadal clumps phagocytic (green) coelomocytes
maturing adults	7-10 grams	immature eggs (25-100μ) sperm not apparent gonadal clumps large coelomocytes (20-40μ) small coelomocytes (5-10μ)
immature worms	0.5-1.5 grams	no sexual products (no gametes or gonadal clumps) large coelomocytes (12-20μ) small coelomocytes (5-10μ)
juveniles	less than 0.1 gram	no sexual products large coelomocytes rare small coelomocytes (5-10μ)

*. Coelomic contents characterized according to descriptions given by Snow (1972) and Snow and Marsden (1974).

neurons in nucleus 20 to react positively in the tests that were used for the demonstration of cystine/cysteine residues, and they pick up labelled cystine more heavily than other cells in this nucleus (Fig. 2), (see below).

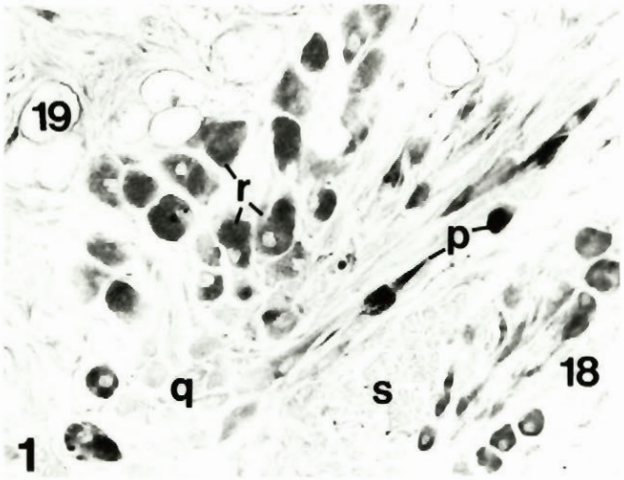
Two other cell types, both PAF-negative, are also present in nucleus 20. Type "q" cells, which predominate in the anterior part of the nucleus, closely resemble the majority of other neurons in the brain (Fig. 1). The fourth type, "s", is characterized by small nuclei, and the possession of little visible cytoplasm (Fig. 1). These cells are found medial to the ventral portion of nucleus 18, and are similar in appearance to the neurons of the corpora pedunculata. The general arrangement of the p, q, r and s cell clusters is diagrammed in Figure 3A,B.

In the brains of immature and juvenile animals PAF-positive cells exist in the same nuclei as in adult ganglia, and the same four cell types, although smaller, are identifiable in nucleus 20 (Fig. 4,5). However, the smaller the brain, the less intense is the positive staining reaction that is observed with PAF (Fig. 5). P cells may not occur at all in the smallest brains, and r cells are very small and stain very palely (Figs. 4,5).

Counts of the nucleus 20 cells have been made in animals at all four developmental stages described in Table 1 (Fig. 6). The PAF-positive types (p and r) show obvious increases in number with increasing maturity. There are approximately 60% more p cells and 80% more r cells in sexually mature animals than in juveniles. The PAF-negative cells (q and s) also seem to increase in number with age, but the changes are less pronounced. There are approximately 50% more q cells and 30% more s cells in fully mature animals than in juveniles. Other PAF-positive neurons in the brain show the same tendencies

Figure 1. Cross section through nucleus 20 of N. virens, stained with PAF (Cameron and Steele, 1959). Four cell types are apparent. Both p and r cells are PAF-positive, while q and s cells are PAF- negative. All ganglionic nuclei are numbered according to Holmgren (1916). 400 X.

Figure 2. Nucleus 20 of an adult brain labelled with ^{35}S-cystine. All cells label, but p cells can be distinguished by their higher grain density. 640 X.



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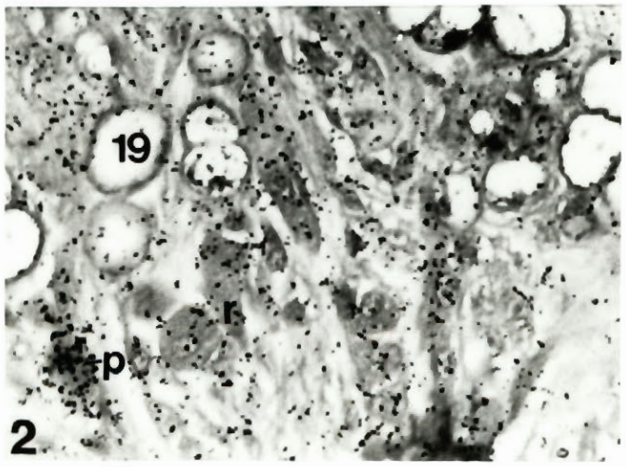

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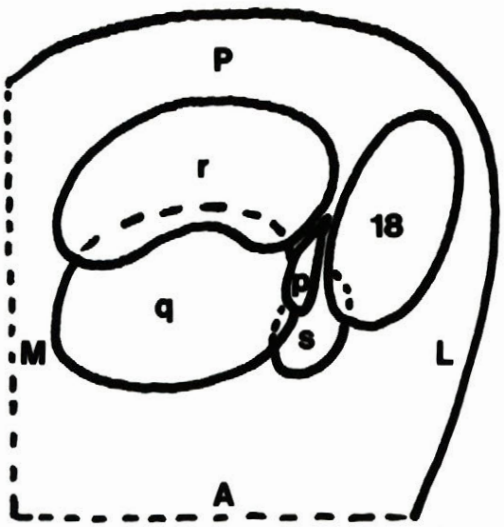
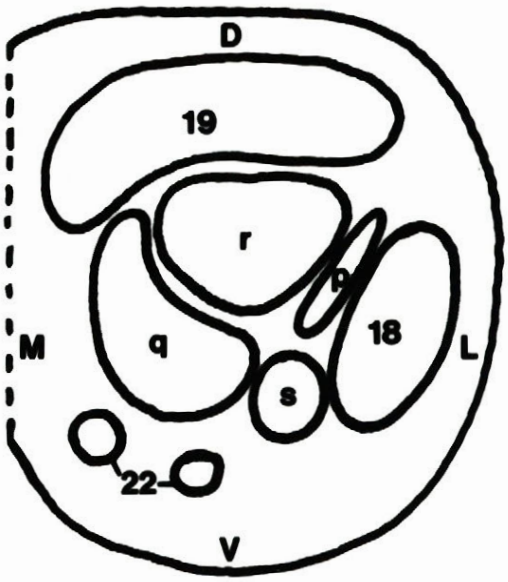
Figure 3. Schematic representations of the relative positions of p,q,r and s cell groups within nucleus 20 of Nereis virens. A-anterior, P-posterior, M-medial, L-lateral, D-dorsal, V-ventral.

3 A. Dorsal view.

3 B. Cross section



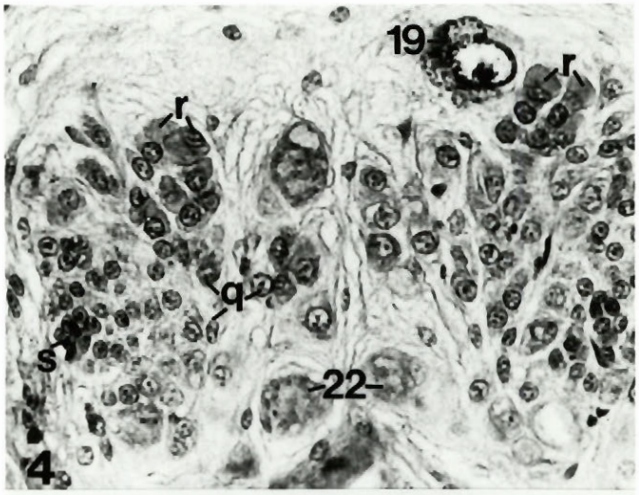
3a



3b

Figure 4. Cross section through nucleus 20 of a small (0.1 gram) specimen of Nereis virens. Three cell types are visible (q,r and s). Hematoxylin and eosin stained. 640 X.

Figure 5. Small N. virens brain stained with PAF (Cameron and Steele, 1959). P and r cells are PAF-positive, but only slightly so. Compare with adult brain, Figure 1. 880 X.


19
r
r
q
s
22
4

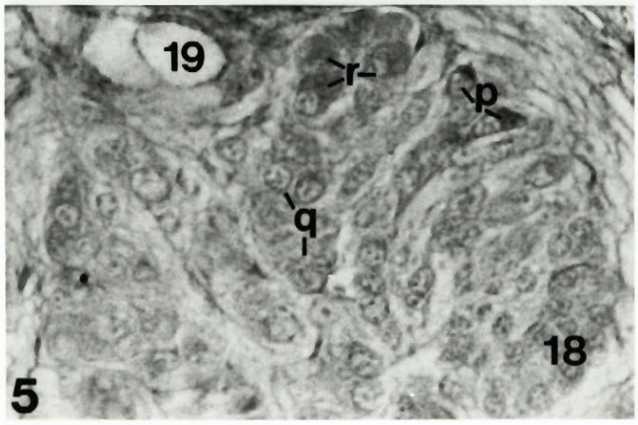
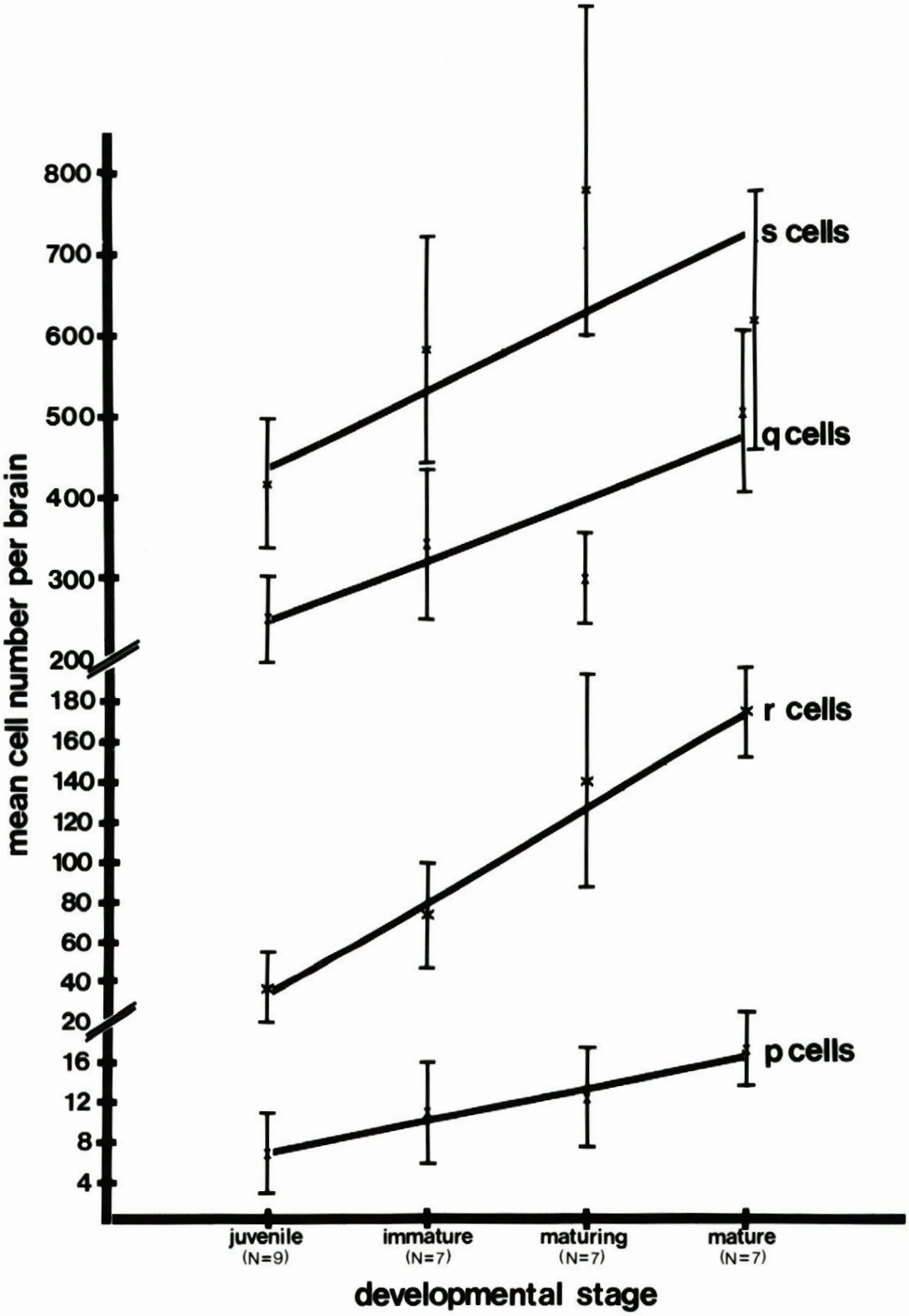

19
r
p
q
18
5

Figure 6. Changes with age in average numbers of four cell types found in nucleus 20 of Nereis virens. The number of brains sampled at each developmental stage is given in brackets along the x-axis (N=..). Nucleus 20 is paired and the values expressed are for both nuclei. Cell types p and r are presumed neurosecretory, types q and s are not.Vertical bars represent standard deviations and lines were fitted by eye.


mean cell number per brain
800
700
600
500
400
300
200
180
160
140
120
100
80
60
40
20
16
12
8
4
s cells
q cells
r cells
p cells
juvenile
(N=9)
immature
(N=7)
maturing
(N=7)
mature
(N=7)
developmental stage

towards increased numbers with age, but since their absolute numbers are low in any case, meaningful quantitative analysis is precluded.

Animals of all ages incorporated ^{35}S-cystine, and all cells in the brain, both neurons and glia, labelled to some degree. Qualitative comparisons between brains revealed that label density increased progressively with longer exposures to the precursor. For any given incubation time, cells in the younger ganglia, except p cells, were more heavily labelled than equivalent cells in older ganglia. Even the oldest brains showed considerable cystine incorporation. As mentioned above, the p cells could be distinguished from other neuronal types in terms of grain density (Fig. 2). This result is consistent with the histochemical evidence indicating that these cells are particularly rich in cystine/cysteine. It is possible, however, that the p cells may be metabolizing at a higher rate in the larger ganglia than in the smaller ones, since these cells first become noticeable in radioautographs of adult brains after incubations of seven hours, but are not distinguishable from neighbouring cells in the smallest brains at incubation times of less than one day.

DISCUSSION

The majority of neurons displaying secretory characteristics at the light microscope level are thus localized in nucleus 20 of the Nereis virens brain. In this study the cellular composition of this entire nucleus has been described for the first time. Four cell types are present, of which two, the p and r cells, may be secretory. These latter cells have been noted in other Nereids by previous investigators - specifically they seem to correspond, in location and staining properties, to the "a" and "c" cells of Scharrer (1937), to nucleus X1XS

of Nereis diversicolor which reportedly contains two cell types (Golding, 1967), and to the type I and II cells, respectively, of Nereis pelagica (Dhainaut-Courtois, 1966). Type I cells are reported to occur in nucleus 7 as well as 20 in N. pelagica, and were suggested as a possible source of the juvenile hormone.

Juvenile hormone content is highest in the brains of immature Nereids, and declines in quantity in maturing worms (Durchon and Porchet, 1971). Neither the p nor the r cells of N. virens seem to fit the pattern that might be expected of cells producing such an hormone. Reaction intensity with PAF[1], and the number and size of p and r cells are all at their lowest in the smallest worms. Maximal colouration with PAF is found in maturing and mature animals (ie. those with sexual products in the coelom), and maximum cell number is achieved in gravid specimens. It therefore may be that the p and r cells produce some hormone(s) associated with events later in the life cycle, such as maturation and/or spawning, rather than juvenile-type hormones. Clearly these cells maintain some metabolic activity in the adult, since they continue to incorporate radioactive cystine at that time of life. Faster labelling by young animals may simply reflect differences in the rate of uptake of material through the body wall due to size differences in the animals, rather than an actual decline in metabolism of the

Footnote

[1]Unlike some other invertebrates, for example insects, in which PAF staining indicates low output of secretory material, active polychaete neurosecretory cells often retain product, therefore are stainable with PAF even while secreting (see Clark, 1965; Clark and Bonney, 1960).

neurons of the older/larger worms. In addition, the p and r cells can be distinguished from each other on the basis of their greater or lesser cystine/cysteine content, so it may be that more than one product is manufactured for secretion by these different cell types.

In N. virens, nucleus 20 considered in its entirety shows higher phosphotidylinositol production in small than in mature brains (Marsden, 1978). This result was interpreted as indicating greater secretory activity in the young ganglia, and thus as supporting the idea that this nucleus might be a source of the juvenile hormone. This notion is not consistent with the present findings. It may be that the heightened phospholipid production in small brains reflects active addition to plasma membrane and/or internal membranes during cell growth, as well as, or instead of the formation of juvenile hormone.

Evidence for the presence of a maturation hormone has been reported in other Nereids. Increased uptake of ^{3}H-cystine by neurosecretory cells was seen in Platynereis dumerilii females bearing developing oocytes in the coelom (Müller, 1973). Labelling declined once the oocytes were mature. Dhainaut-Courtois (1966, 1968) has noted in passing that the neurosecretory type I and II cells in Nereis pelagica and N. diversicolor reach maximum numbers in animals just beginning to develop gametes. Contrary to the above observations on Nereis virens, no further changes in cell number were reported in fully mature worms, although detailed cell counts were not presented.

In summary, this morphological-radioautographic study describes, for the first time, the entire cellular composition of ganglionic nucleus 20 in Nereis virens. Two of four occurring cell types, p and r cells, appear to be secretory in nature. Both p and r cells increase

in PAF staining intensity, in size and in number with age, reaching a maximum at sexual maturity. The role of these cells is purely speculative at the moment, however, they might well produce an hormone associated with gamete maturation, such as Müller has described in Platynereis dumerilii, or one inducing spawning, such as exists in certain Nephtyidae (Olive, 1977). The possibility that these cells might be secreting hormone(s) throughout the life cycle has not been eliminated. In any case, since this study suggests that humoral control over maturation may be more elaborate than initially suspected, at least in Nereis virens, it has been used as a basis for two further morphological studies, on the ultrastructure of this nucleus, and on nervous system/neurosecretory cell development in this species (see Chapters 4 and 6, this thesis). It is also hoped that this description will be of use in future comparative studies of neurosecretion in the Nereidae.

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CHAPTER 4

THE ULTRASTRUCTURE OF GANGLIONIC NUCLEUS 20 IN THE BRAIN OF *Nereis virens* (POLYCHAETA). AGE-RELATED CHANGES IN NEUROSECRETORY CELLS

ABSTRACT

Ultrastructural examination of the four previously described cell types within ganglionic nucleus 20 in the hindbrain of Nereis virens confirms that two cell types, p and r, may be neurosecretory. In adult worms they are packed with elementary granules, p cell granules averaging 1280 A in diameter, r cell granules 1720 A. The ultrastructure of both cell types, and their continued prominence into sexual maturity supports the idea that they are functional in the adult. R cells show a depletion of granule content, suggestive of secretion, in animals which have spawned, so it may be that they produce a hormone inducing spawning. P cells show no discrete burst of secretion, and may, therefore, produce a trophic hormone which is required throughout the period of sexual maturation. However, both these cell types arise early in development, and have the ultrastructural appearance of functional neurosecretory cells at that time, so it is likely that they are actively secreting in juvenile animals also. It is suggested that these cells may represent a source of hormones throughout the life cycle of this species.

INTRODUCTION

A juvenile hormone (or hormones) is known to emanate from the brain of sexually immature Nereids. This factor, or possibly a group of factors, facilitates growth and regeneration, and inhibits sexual maturation. A natural decline in the amount of controlling hormone with age is followed by the cessation of growth and regeneration, and the onset of reproductive changes. (See Baskin, 1976; Clark, 1965; Golding, 1974 for reviews.) Although a cellular source of such hormones has never been located, numerous neurons which might be of secretory significance have been described in the brain on the basis of light and electron microscope studies.

For example, Golding (1967) has found cells containing neurosecretory-like granules in 22 of the 26 ganglionic nuclei of the brain of Nereis diversicolor. He attributes possible secretory function to cells of nucleus 20 (X1XS) and 22, in which the dense granules and rough endoplasmic reticulum are especially abundant. Also Dhainaut-Courtois (1966, 1968) has described six cell types in the brain of Nereis pelagica, of which she believes types I and II neurons may be secretory. Type I cells are reported to occur in nuclei 7 and 20, and to reach predominance in animals lacking genital products. These cells have thus been suggested as a source of juvenile hormones. Type II neurons reach maximal number in animals developing gametes, and are of unresolved function.

In a previous light microscopic study (see Chapter 3 of this thesis) four neuronal types (p, q, r and s) were described in nucleus 20 of the Nereis virens brain. Types p and r, which appear to correspond to types I and II respectively, are also apparently neurosecretory. However, unlike in Nereis diversicolor, these cells in N. virens

are most prominent in terms of number, size and paraldehyde fuchsin (PAF) staining intensity, in sexually mature adults. It was therefore suggested that they might secrete products associated with events late in the life cycle of this Nereid, such as sexual maturation or spawning.

Nucleus 20 has since been examined by electron microscopy. The ultrastructure of the four cell types constituting this nucleus is presented, and age-related changes in the p and r cells, which might be related to secretion, are described. The possible role of these cells in the N. virens life cycle is further discussed.

MATERIALS AND METHODS

Specimens of Nereis virens, of all ages, were obtained from Marine Research Associates, Ltd., Deer Island, New Brunswick. Spawning adults were collected at Deer Island during the 1977 and 1978 spring breeding seasons. The worms were classifed, as described previously (see Chapter 3 of this thesis), on the basis of weight and degree of sexual maturity. Supraoesophageal ganglia were dissected from animals representative of each stage of maturation, and fixed two to four hours at room temperature in 4% glutaraldehyde in 0.2M sodium cacodylate buffer (pH=7.9). The tissue was washed one hour in 0.2M sodium cacodylate, then post-fixed two hours in 2% osmium tetroxide in the same buffer. All solutions contained 5% sucrose, and one drop per 10 mls of both 1% magnesium chloride and 1% calcium chloride. This fixation method was adapted from Baskin (1971) and Marsden and Lacalli (1978).

Brains were Epon embedded and sectioned on a Sorval MT2 ultramicrotome. Alternating semi-thick (0.5μ) and thin sections were taken. Semi-thick sections were stained for light microscopy with paraldehyde fuchsin (Steel and Morris, 1977) and counterstained with a 70% ethanolic preparation of Halmi's trichrome (Halmi, 1952).

Corresponding thin sections were stained with 4% uranyl acetate in 40% ethanol, and lead citrate (Reynolds, 1963), and viewed on a Philips EM 200. Measurements of subcellular organelles within the various neuronal types were made from electron micrographs at magnifications of approximately 9,000 to 45,000 times. The numbers of existing Golgi profiles and lysosomal bodies in p and r cells were counted and cytoplasmic areas were determined from the micrographs by the use of a planimeter. The area occupied by rough endoplasmic reticulum in these cells was estimated by linear integrative analysis (Loud, et al, 1965), and the mean diameters of their elementary granules were calculated according to the method of Giger and Reidwyl (1970).

RESULTS

The four cell types known to exist in nucleus 20 were identified under the electron microscope by direct visual comparison of thin sections with corresponding PAF stained semi-thick sections. Only p and r cells are PAF positive, q and s cells are not (Fig. 1). The relative positions of these four cell types within nucleus 20, and their appearances under the light microscope, have been previously described (see Chapter 3 of this thesis).

Ultrastructure of the q and s Cells in Maturing and Fully Mature *Nereis virens*

S cells are the smallest cells in this ganglionic nucleus, being about 3 microns in diameter. The bulk of the cellular area is occupied by the nucleus. Small quantities of rough endoplasmic reticulum exist, especially around the nucleus, while the other organelles are scattered throughout the cell body. These cells contain low numbers of dense granules, ranging in size up to 700 A, and small lucent vesicles up to

500 A, in diameter (Fig. 3).

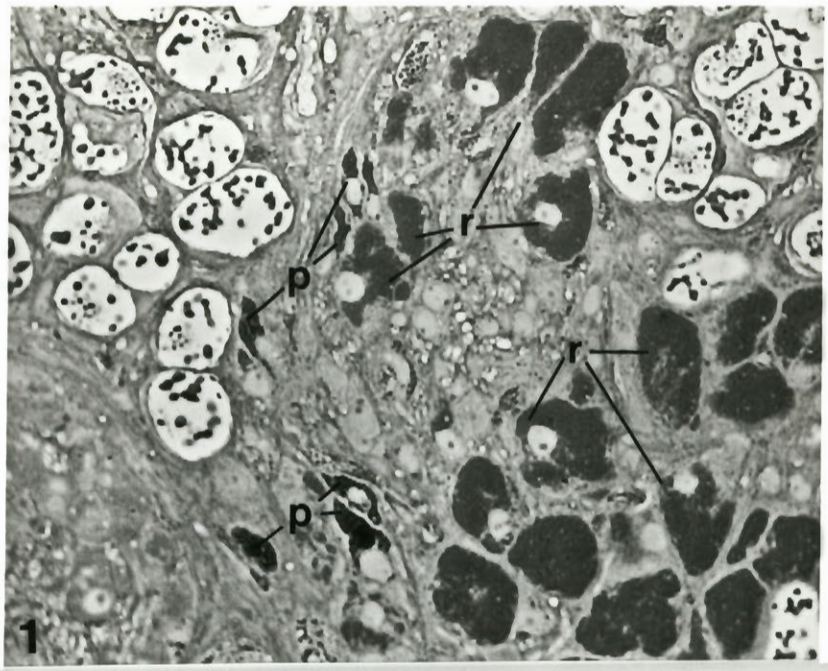
Q cells resemble s cells in general ultrastructure, although they are larger (8-10 microns in diameter), and a higher proportion of the total cellular area is cytoplasmic. There is much variation in both the size and the quantity of granular inclusions found within these cells. A few small clear vesicles, ranging in diameter up to 700 A, and some dense granules, ranging in diameter up to 1100 A are seen (Fig. 2).

Ultrastructure of the p and r Cells in Maturing and Mature Animals

The p and r cells are ultrastructurally similar, although they differ in shape (p cells are fusiform, r cells pyriform), and location, as previously described (see Chapter 3 of this thesis). Both cell types contain rough endoplasmic reticulum arranged, primarily, around the nucleus and beneath the plasma membrane. Golgi profiles and lysosomes or multivesicular bodies are not common, but do appear to be randomly distributed throughout the perikarya. Small numbers of electron lucent vesicles, up to 800 A in diameter, are sometimes seen in the vicinity of Golgi bodies. However, the most prominent feature of both cell types is the abundance of large dense granules within their cytoplasm (Figs. 4,5). These granules are membrane-bound and are sometimes seen arising from the Golgi apparatus. The mean diameter of such granules is 1720 A in the r cells, and 1280 A in the p cells of maturing adults, and 1940 A and 1750 A respectively, in the r and p cells of fully sexually mature adults. A visible depletion in the number of stored granules is seen in the r cells of fully mature specimens immediately after spawning has taken place (Fig. 6). At all other stages of the life cycle these cells are replete with granules. None of the other cell types change in appearance at that time.

Figure 1. Cross section through nucleus 20 of the adult N. virens brain. Epon stained with paraldehyde fuchsin and alcoholic Halmi's. The PAF-positive p and r cells are shown. 640X.

Figure 2. Q cell of maturing adult. 17,200X.


r
p
r
p
1

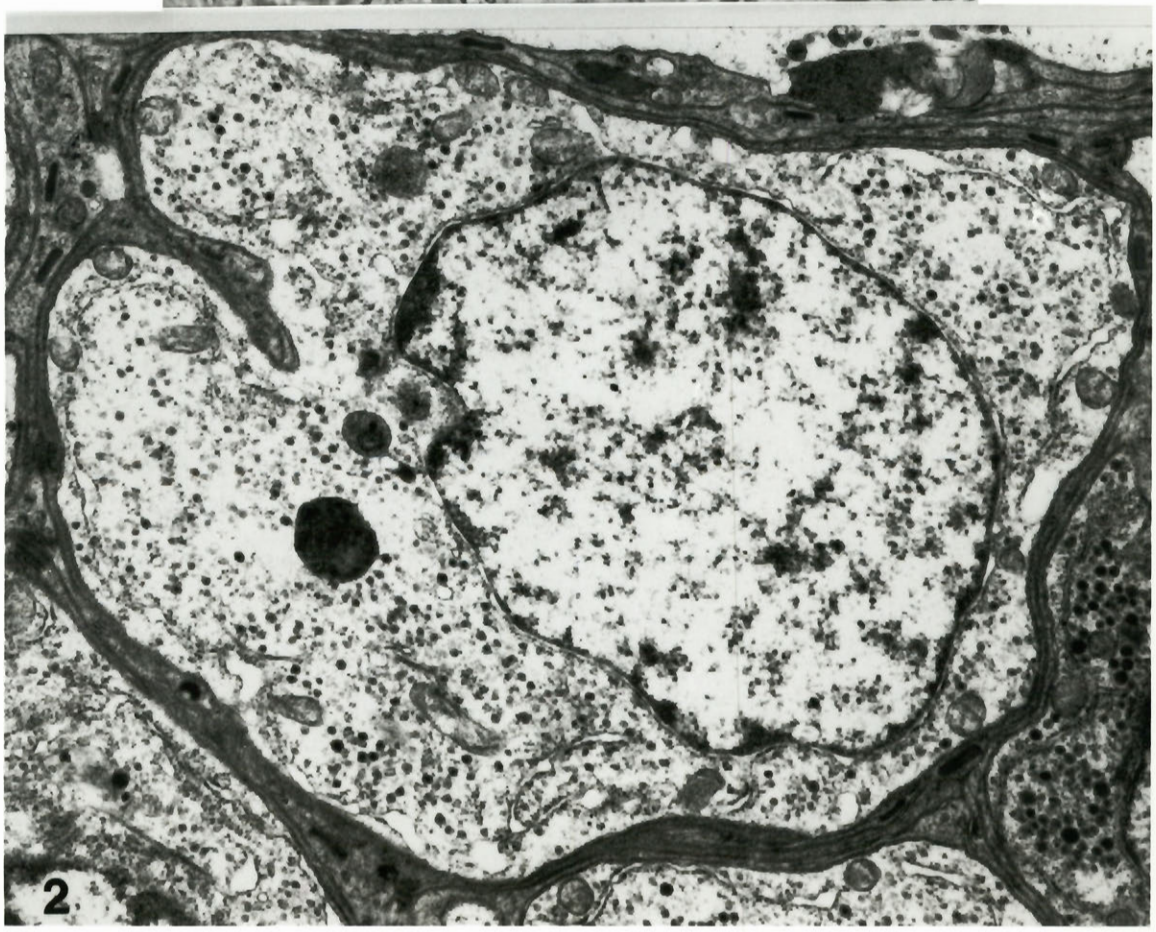

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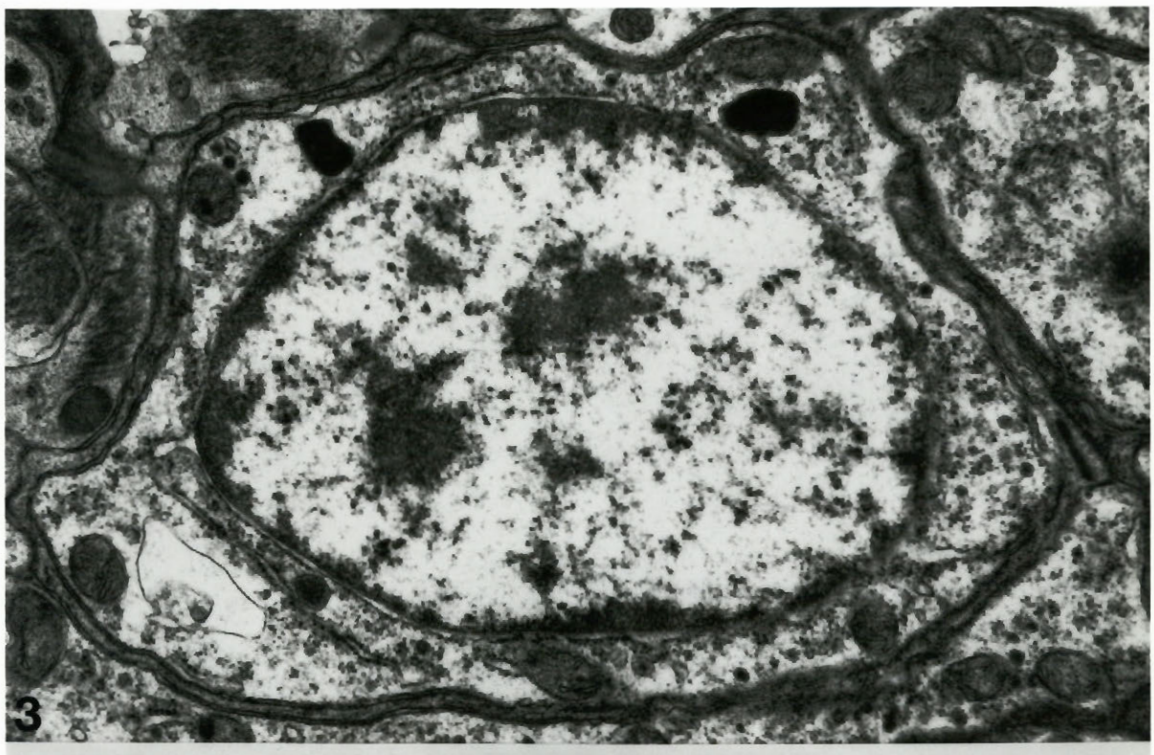
Figure 3. S cells of maturing adult. 38,500X.

Figure 4. P cell of maturing adult. Elementary granules fill the cell. 38,500X.


3

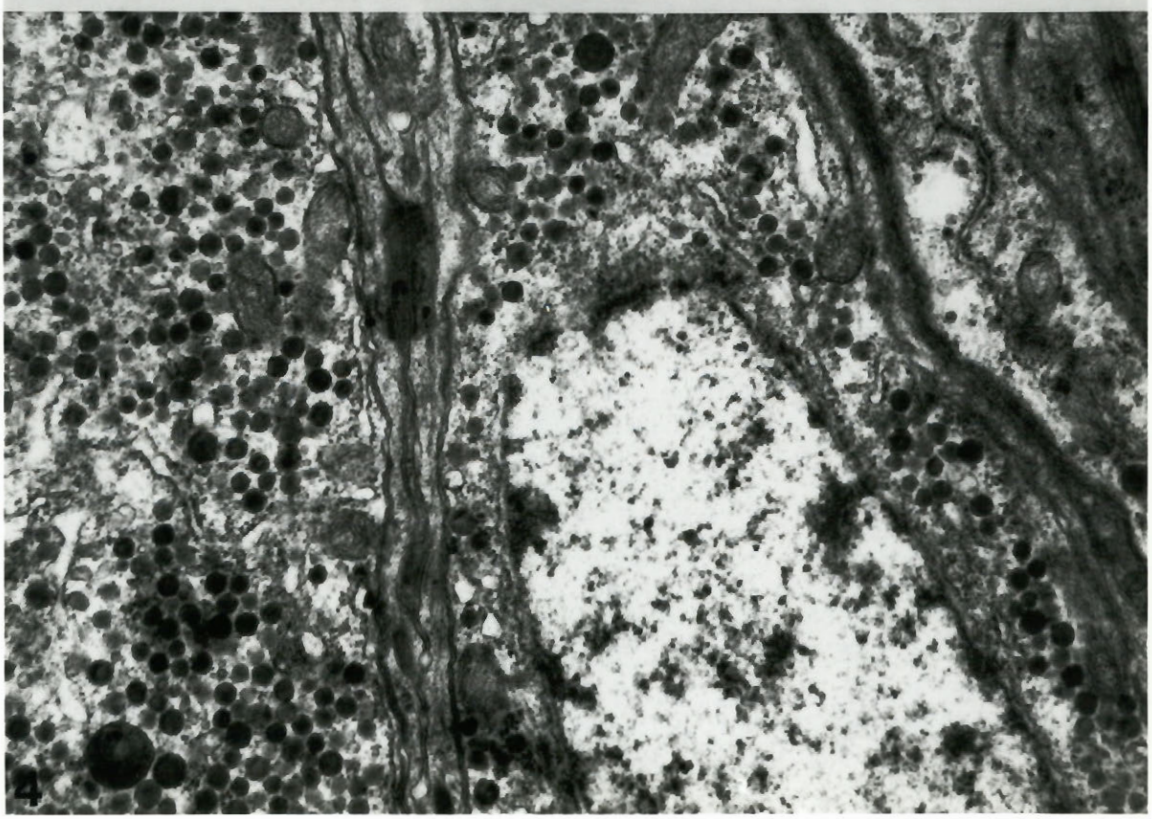
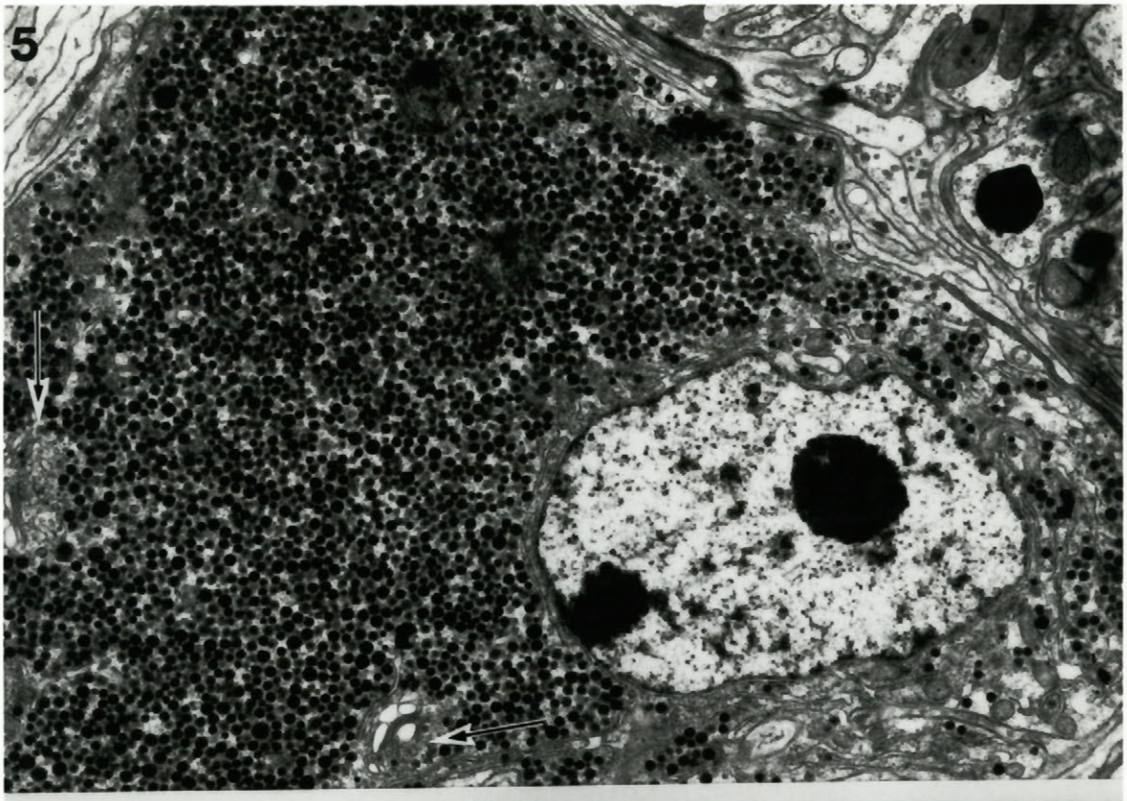
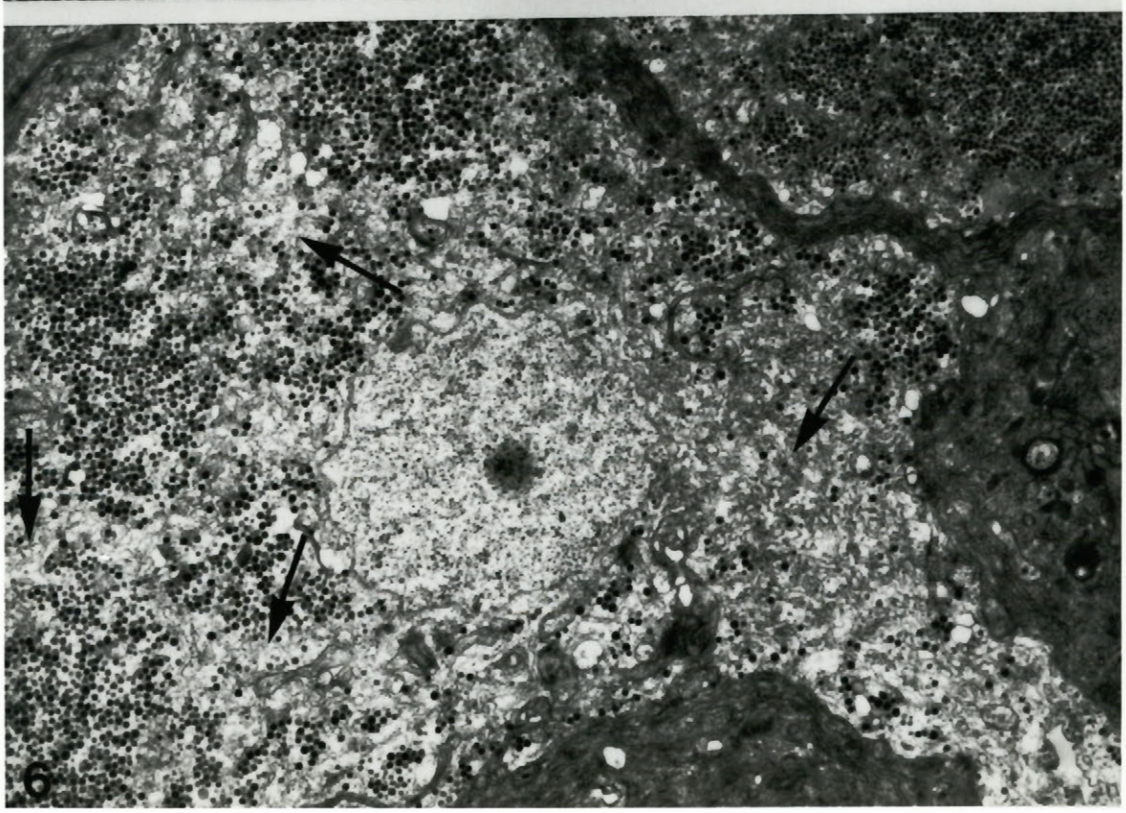

4

Figure 5. R cell of maturing adult. Note profusion of dense granules throughout the cell, and occurrence of active Golgi bodies (arrows). 10,200X.

Figure 6. R cell of sexually mature adult fixed after spawning. Note that certain areas of the cytoplasm are now empty of secretory granules (arrows). Compare with Figure 5. 6,050X.


5


6

Ultrastructure of the p and r Cells in Sexually Immature and Juvenile Animals

These two cell types are identifiable in nucleus 20 throughout the life cycle, except early in development, before the brain is fully differentiated. R cells first appear at 2-3 months of age, in juveniles numbering 25 to 30 segments, while p cells appear at 9-10 months of age, in juveniles of 70 to 80 segments. These young cells closely resemble adult p and r cells in ultrastructure. Juvenile as well as mature cells were seen to contain active golgi bodies and large numbers of elementary granules.

Changes noted with age in the number of granules, Golgi profiles, and lysosomes, and in the area occupied by rough endoplasmic reticulum, are summarized in Table 1. There were no consistent differences in the occurrence, throughout development, of the various synthesizing organelles in the p cells, but r cells showed small decreases in the numbers of Golgi bodies and lysosomes with age. Both cell types showed decreases in the number of presumptive secretory granules per unit area but increases in the size of these granules with age. R cell granules in sexually mature worms are approximately 70% larger than those in juveniles, while the p cell granules are about 80% larger at sexual maturity than during juvenile life (Table 1).

DISCUSSION

The above observations seem to support the notion that the PAF-positive p and r cells of the Nereis virens brain may be neurosecretory. They are packed with large electron dense granules resembling the classical elementary granule of secretory cells and are ultrastructurally very similar to apparently analagous cell types in other Nereids which have been presumed neurosecretory (Dhainaut-Courtois, 1968; Golding, 1967). The p and r cells differ

Table 1

Age-related changes in the Golgi profiles, lysosomes, rough endoplasmic reticulum and elementary granules of the p and r cells. Variations are expressed as standard deviations, and the numbers of cells sampled at each developmental stage are given in brackets (N).

developmental stage*	Golgi profiles per 100 ^{2} of cytoplasm	lysosomes per 100 ^{2} of cytoplasm	of rough ER per 100 ^{2} of cytoplasm	granules per 100 ^{2} of cytoplasm	mean granule diameter (A)
p cells					
juvenile (N=5)		insufficient samples		225±50	1940±70
immature (N=10)	4.0±1.7	7.0 ±1.4	94±35	2075±76	1280±163
maturing adult (N=10)	1.9±0.9	10.7 ±1.7	170±32	1729±53	1280±82
fully mature adult (N=10)	3.5±1.2	9.0 ±4.9	73±17	1642±33	1750±47
r cells					
juvenile (N=17)	1.7±0.8	3.3±1.8	120±40	4289±235	330±196
immature (N=20)	1.4±1.1	2.3±1.4	86±20	2663±87	1630±233
maturing adult (N=20)	0.8±1.7	1.2±1.4	100±35	2407+25	1720±98
fully mature adult (N=20)	0.6±1.1	1.1±1.6	75±15	1884±24	1940±176

* The animals were classed according to weight and the degree of sexual maturity, as previously described (see Chapter 3 of this thesis).

ultrastructurally, for example, in terms of granule size, as well as histochemically (see Chapter 3, Appendices I, II), and therefore may be presumed to manufacture different materials.

The continuation of synthetic activities by both these cell types, as indicated by the presence of granules, rough endoplasmic reticulum, and Golgi profiles elaborating granules, throughout adulthood and into sexual maturity, as well as their peak in numbers and size at that time (see Chapter 3 of this thesis) supports the suggestion that both p and r cells are of functional importance during the latter part of the life cycle of this species. Synthesis may slow down in these cells with age (note decreases in granule content and numbers of Golgi profiles), but it does not stop. Nor do any of the mature cells show signs of increased degradation or autolysis such as might be expected in regressing cells. Such changes were reported by Marsden (1978) in sexually mature N. virens males, in cells she believed to belong to nucleus 20 (X1XS), however, the cells she has described resemble certain neurons found in the nuchal nucleus (type III, Dhainaut-Courtois, 1968), more closely than the p or r cells, therefore they may have been misidentified.

The morphology of the r cells immediately after spawning suggests that those cells in particular may secrete large quantities of material at that time. R cell product might therefore be equivalent to the hormone which induces spawning in Nephtys hombergi (Olive, 1977). P cells show no discrete depletions of their granule content, thus the timing of their secretory cycle remains unclear. It is possible that these cells secrete some sort of "trophic hormone", perhaps related to gamete development or maintenance, which is required throughout adulthood. The adult brain is known to exert a positive influence on sexual development in some Nereids. For example, Müller

(1973) has described a "maturation" hormone in Platynereis dumerilii, the secretion of which is correlated with oocyte ripening. Increased uptake of tritiated cystine by certain neurosecretory cells in the brain was noted in females bearing immature oocytes, while labelling declined once the oocytes had reached maturity.

From this study it would appear that p and r cells are not active only in adults. They present the ultrastructural appearance of functional neurosecretory cells from the time they are first recognizable in the brain. At that time they may even exceed the adult cells in numbers of Golgi bodies, quantities of rough endoplasmic reticulum and stores of secretory granules. These cells therefore may be secreting products in the juveniles as well as in the adults, and might conceivably be a source of "juvenile hormone", assuming this hormone to exist in N. virens as it does in other Nereids. This raises the possibility that p and r cells manufacture more than one hormone during their life span. The observed changes with age in granule diameter in both these cell types might indicate plurality of products.

It has been assumed here that the appearance of the neuronal perikarya reflects events at the site(s) of hormonal release. Since the p and r cells maintain a relatively constant ultrastructure throughout life, and since changes which might be indicative of rapid or short-term secretory cycles are not observed, it would seem a reasonable conjecture that an equilibrium is being maintained between a fairly slow manufacture and a slow but constant release of product. The r cells would seem to diverge from this equilibrium at spawning time, and to secrete product in excess of its synthesis. Final determinations of the

timing of secretions by these cells will require examination of their terminations, which have not to date been identified. However, the combined results of this study, and a previous light microscopic study of the same nucleus (see Chapter 3 of this thesis), suggest a more extensive involvement of the p and r cells in hormonal control of the life cycle of N. virens than has ever been attributed to analagous cells in other Nereids. Further experiments, both morphological and physiological (for example decerebration and brain replacement experiments), and especially studies of a comparative nature, will be required for complete clarification of the role of such neurons in growth and development.

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CHAPTER 5

PRELIMINARY EXPERIMENTAL EVIDENCE FOR A SPAWNING HORMONE IN *Nereis virens* (POLYCHAETA).

ABSTRACT

A crude homogenate prepared from brains of spawning Nereis virens was observed, when injected, to induce spawning in a small proportion of gravid recipients. Sea water injected control animals showed a lesser effect. This experiment, in conjunction with previously described morphological evidence, suggests that an hitherto unrecognized hormonal control over spawning may exist in at least this Nereid.

INTRODUCTION

Numerous past experiments (for reviews see Baskin, 1976; Clark, 1965; Golding, 1974) on various members of the Nereidae provide evidence for a "juvenile hormone" in this family. This hormone, or possibly a group of hormones, originates in the immature brain (supraoesophageal ganglion) and stimulates growth and regeneration while simultaneously inhibiting sexual maturation. A decline in inhibitory activity of the brain occurs with age (Durchon and Porchet, 1971) and thus allows the onset and normal progression of all processes associated with sexual maturation. However, evidence for additional hormonal activity by the adult brain has also been uncovered. Müller (1973) has described increased activity by certain neurosecretory cells in the brains of female Platynereis dumerilii parallelling oocyte maturation. Presumably a "maturation hormone" stimulates oocyte growth in some fashion.

Likewise, histological and ultrastructural studies of Nereis virens have led to the suggestion that certain putative neurosecretory cells in the brain of this species continue activity throughout the life cycle (see Chapters 3 and 4, this thesis). Two neuronal types, p and r cells, which occur in the hindbrain, reach maximum prominence in terms of number, size, and paraldehyde fuchsin staining intensity in adult specimens. These cells maintain the ultrastructural appearance of functional neursecretory cells throughout sexual maturation, and the r cells show a notable decrease in granule content, suggestive of secretion, in association with spawning. It was therefore suggested that a hormone inducing spawning might be present in this species.

Olive (1977) has described a series of experiments which indicate that such an hormone exists in the Nephtyidae. Homogenate prepared

from brains of mature animals was seen to induce spawning when injected into certain experimental animals. Controls injected with sea water, or extract prepared from suboesophageal ganglion, did not spawn to any significant extent.

A similar experiment has been performed on Nereis virens, in an attempt to determine if a spawning hormone does exist in this species, and the results are reported here.

MATERIALS AND METHODS

Sexually mature Nereis virens adults were collected during the 1979 breeding season, the majority being supplied by Marine Research Associates, Ltd., Deer Island, New Brunswick. Two groups of sexually mature animals were available for experimentation - one group of thirty males obtained in early May, and one group of twenty-one females obtained in mid-June.

When the animals were subjected to laboratory conditions, involving handling, as well as warmer temperatures, some were observed to begin spontaneous spawning. These worms were isolated for use as donors. Their brains were dissected out as rapidly as possible, and homogenized individually in sea water in a Wheaton micro tissue grinder (0.1 ml capacity). The homogenate from one donor brain was diluted to 0.5 ml with sea water and immediately injected into a non-spawning recipient. Control animals were injected at the same time with 0.5 ml of sea water. All animals were subsequently observed for one hour for signs of spawning behaviour and gamete release.

RESULTS

All animals showed active swimming behaviour after being injected, however, swimming was not always accompanied by spawning. Three of ten experimental males were observed to shed sperm through the pygidium, but none of the ten sea water injected controls released any sperm. Four of seven females injected with brain extract shed oocytes, as did two of seven control females. There was large variation in the number of oocytes that were released by the individual females.

All spawnings that were observed took place within ten minutes of injection in both control and experimental groups.

DISCUSSION

Such results offer some support for the idea, previously derived from morphological studies (see Chapters 3 and 4, this thesis), that the brain of Nereis virens performs some secretory role in sexually mature adults.Since a higher proportion of animals were observed to spawn after injections of brain extract taken from spawning donors, than after sea water injections, it is suggested that the brain of gravid adults may be the source of a substance which has the capacity to induce spawning. This putative neurohormone might resemble the spawning hormone of Nephtys hombergi (Olive, 1977), which is thought to control the body wall musculature during the process of gamete emission. It probably would not resemble the "maturation hormone" of Arenicola marina, which is necessary for the induction of the final maturation divisions of the ova and the separation of sperm from morulae immediately preceding spawning in that species (Howie, 1963, 1966). In N. virens gametes ripen in the body cavity over a period of several months (Snow, 1972;

Snow and Marsden, 1974), rather than abruptly, prior to spawning, as in Arenicola.

Not all experimental animals were observed to respond to injections of brain homogenate. N. virens is monotelic, but appears to spawn more than once during the breeding season (Bass and Brafield, 1972). Therefore it may be that the success of an injection depends on the state of the recipient, for example whether it is still capable of spawning at all, or whether it is close to being spent, as well as on the state of the donor. The quantity of "spawning hormone" within the donor ganglion may well be decreased after repeated spawnings. These findings are only preliminary, since worms were not available in sufficient numbers to permit further sampling or reliable statistical analysis. Should confirmatory evidence be obtained, other interesting questions will remain to be answered, on the exact localization of this substance within the brain, on the mode of action of this "hormone", and on the similarities and/or differences between male and female brains in these regards.

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CHAPTER 6

THE MORPHOLOGY AND DEVELOPMENT OF THE LARVAL NERVOUS SYSTEM IN *Nereis virens* (ANNELIDA, POLYCHAETA)

ABSTRACT

The morphology and behaviour of Nereis virens larvae, grown in culture, have been studied. Clumps of undifferentiated cells located laterally in the epidermis are seen to give rise to the rudiments of the adult nervous system, including the brain, the ventral nerve cord and corresponding neuropil tissue, within 4 days of fertilization. The first differentiated neurons, with processes, are small rounded cells rich in glycogen and containing a variety of lucent vesicles and dense granular inclusions. A second cell type, with densely staining nucleus and cytoplasm, also exists, and appears to play the role of a glial cell or a fibrocyte by separating the nervous system cells from surrounding non-neural tissue. The connective tissue capsule of the brain is completed only after hatching, and the cells within the brain continue to divide or differentiate, with the net result being a gradual increase in brain size. This parallels body growth, which is gradual as well, involving the addition of new segments and the enlargement of existing ones. Among the last neurons to differentiate are those in certain ganglionic nuclei recognized as neurosecretory in the adult. It may be that brain hormones play no role in the larval portion of the life cycle of this Nereid. Behaviour of hatchlings includes swimming by the use of the trochal bands of cilia, and crawling by use of the parapodia and body musculature. Possible en passant contacts were seen between nerve processes and circular, longitudinal and parapodial muscles, but no nervous connections with the ciliated cells were found. Experiments with various neurotransmitters indicate the involvement of acetylcholine, serotonin and glutamic acid in neuro-muscular control in these larvae.

INTRODUCTION

The brain of the young Nereid is of documented importance as a source of "juvenile hormone"(s) which regulate(s) the processes of growth and regeneration and repress(es) sexual maturation. (For reviews see Baskin, 1976; Golding 1972, 1974; Golding and Whittle, 1977.) Putative neurosecretory cells have been identified in grown specimens of several species (Dhainaut-Courtois, 1966, 1968a; Golding, 1967; Chapters 3 and 4 of this thesis). However, little information is available on the histology or ultrastructure of Nereid larvae, or the larval brain, and nothing is known about existing hormonal controls over this part of the life cycle. In this study larvae of Nereis virens, raised in culture, have been examined at various developmental stages. The structure of the developing nervous system, in particular the brain, is described, and possible functions of the larval nervous system, including neurosecretion, are discussed.

MATERIALS AND METHODS

Fertilized eggs were obtained from spontaneous laboratory spawnings of gravid adults collected at Deer Island, New Brunswick, during the 1977, 1978 spring breeding seasons. Until hatching was completed, batches of a few hundred eggs each were kept in sea water in 200 ml disposable plastic beakers. Sea water was replaced daily. Hatched larvae were transferred to aerated glass fish bowls (approximately two liter capacity) with a small amount (roughly two millimeters) of fine mud in the bottom, and were fed 5 mls of assorted algal cultures every two days. Hatchlings of 3 to 5 setigers were fed small species such as Isochrysis galbana, Dunaliella salina, and Amphidinium sp., but a switch was made to larger species such as Ochrosphaera neopolitana and

Cyclotella meneghiniana as the larvae grew. Animals were kept at 11-15°C.

Behavioural observations were made on the cultures as they developed and the effects of several neuropharmacological agents (acetylcholine, carbachol, atropine, serotonin, noradrenaline, adrenaline, eserine sulfate, glutamic acid, and gamma-amino butyric acid) on 3 setiger hatchlings were noted. All transmitters were mixed fresh, at a concentration of 10^{-2}M, in sea water. Groups of larvae were isolated in ceramic spot plates. The sea water around them was pipetted off, and replaced by one of the transmitter solutions. After a few minutes observation, the animals were returned to plain sea water. When recovery seemed complete the experiment was repeated.

Animals were sampled for both light and electron microscopy at various developmental stages from pre-hatching through to 60-70 setigers. For light microscopy larvae were fixed in Bouin's fluid and paraffin embedded. Five micron sections were stained with either hematoxylin and eosin, or paraldehyde fuchsin (Cameron and Steele, 1959). Certain animals were labelled for radioautography with tritiated thymidine (New England Nuclear, S.A.= 50 Ci/mM, concentration= 0.0024 mg/ml) by incubating them in sea water containing 1μCi/ml of that precursor. Labelled larvae were fixed in Bouin's fluid, and embedded in paraffin. Five micron sections were prestained with Delafield's hematoxylin and eosin, then coated with Kodak NTB2 bulk emulsion. Radioautographs were developed 6 minutes in D170 and fixed 3 minutes in 24% sodium thiosulphate (Kopriwa and Leblond, 1962).

Larvae for electron microscopy were fixed one hour at room temperature in 4% glutaraldehyde in 0.2M sodium cacodylate (pH=7.9) washed 30 minutes in 0.2M sodium cacodylate, and post-fixed one hour in

2% osmium tetroxide in the same buffer. All solutions contained 5% sucrose and one drop per 10 mls each of 1% calcium chloride, and 1% magnesium chloride. Material was dehydrated in ethanol and propylene oxide and embedded in Epon. Alternating thick (0.5 micron) and thin sections were cut on a Sorvall MT-2 ultramicrotome. Thick sections for light microscopy were stained with either 1% toluidine blue in 1% borax, or in paraldehyde fuchsin (PAF) (Steel and Morris, 1977) followed by a 70% ethanolic preparation of Halmi's trichrome (Halmi, 1952). Thin sections were stained three minutes with 4% uranyl acetate in 40% ethanol, and two minutes with Reynolds' lead citrate (Reynolds, 1963), and viewed on a Philips EM 200.

RESULTS

General Growth

By three days post-fertilization gastrulation is completed, and the embryos, enclosed in a transparent gelatinous envelope roughly 100 microns thick, are recognizably polarized in a rostro-caudal plane. The head is distinguished by a red pigment band associated with the developing prototroch. By the fourth day post-fertilization setal sacs with setae are present, although the bristles remain internalized beneath the larval cuticle. Three sets of setae develop simultaneously. A pharynx begins to develop, although the rest of the gut is undifferentiated and yolky cells fill the bulk of the body cavity. During the fifth and sixth days post-fertilization the setae grow out through the larval cuticle and a pair of eyespots develop.

Larvae hatch at seven days post-fertilization, still as 3-setigers. Palps are beginning to grow, as are two pairs of tentacular cirri on the first segment, and teeth within the pharynx. Thereafter the larvae elongate rapidly by the addition of segments between the last existing

segment and the pygidium, at the rate of about one segment every one to two days. The animals reach 6-setigers by 14-15 days post-fertilization, by which time the gut is differentiated, the yolk complement is depleted, and rudiments of all adult head appendages are present. Growth then slows to a rate of about one segment every three to four days. Within 50 days of fertilization larvae achieve 20-30 setigers. Growth to adult size (about 150 segments) continues by segment addition and enlargement of existing segments.

Behaviour

In unhatched red pigment band larvae cilia of the trochal bands are observed beating, and muscular actions include contractions of the gut, slight twitches of the parapodia, and protraction/retraction of the growing setae. Once animals have hatched, they swim by use of their cilia, holding the setae close to the body as they do so, but they also crawl actively, using the parapodia as paddles, and bending the body from side to side. For a few hours immediately after hatching the animals display a positive photo-taxis, and will swim to the surface or side of the culture dish if a light is applied. Thereafter, they stay close to the bottom of the dish, and often are noted to adhere to the substrate by their heads. They are seen feeding on available sediment and particles, and will burrow in fine silt.

Neuropharmacological Effects

Noradrenaline, adrenaline, eserine sulfate and GABA had no effect on the larvae. Normal swimming and crawling behaviour continued apparently unchanged in the presence of these agents. Acetylcholine, carbachol, serotonin and glutamic acid all had similar positive effects.

Animals stopped moving and remained arrested with parapodia directed straight outwards. Acetylcholine acted immediately and had the strongest effect. In the presence of this drug animals did not move at all. Carbachol, serotonin and glutamic acid acted more gradually than acetylcholine, and animals were capable of twitching parapodia or setae if touched. Recovery from these four drugs followed the same pattern. Larvae exposed to carbachol, serotonin or glutamic acid resumed spontaneous movements and crawling within a minute or so of removal of the transmitter, while the effects of acetylcholine were prolonged for five to ten minutes following its replacement with sea water. In addition to a generalized arrest, atropine appeared to produce gut contractions. Animals exposed to atropine were observed to extrude the yolky contents of the gut, and subsequently died. None of the various chemicals were noted to influence movement of the cilia.

Nervous System Development in Unhatched Larvae

Morphological examination was begun at the red pigment band stage. The earliest RPB larvae (three days post-fertilization) do not appear to have any organized nervous system. Numerous undifferentiated cells occur in the epidermal region, with especially noticeable clumps situated laterally in the head. These cells, all similar, are rounded, with no processes, possess only limited quantities of cytoplasm, and are quite small (2 to 4 microns in diameter). They are rich in what appears to be glycogen, and some are seen to label with tritiated thymidine in radioautographs (Fig. 1), therefore probably are dividing. As the red pigment band stage progresses, the basic structure of the adult nervous system is laid down. By four days post-fertilization

undifferentiated cells, resembling those in the lateral clumps, appear beneath the epidermis in the dorsal part of the head, in the area which will be occupied by the adult brain (Fig. 2), and in the region of the developing ventral nerve cord. However, some of the nervous system cells have differentiated visible processes by this time (Fig. 3), and fibrous neuropilar material is apparent in the vicinity of the brain and nerve cord. Fibres range in diameter from 0.2 to 1 micron, and contain dense granules, ranging up to 800 A in diameter, and electron lucent vesicles, ranging up to 500 A in diameter. Such particles are also found in the somata of the first neurons presumably indicating that the manufacture of such materials as neurotransmitters has begun (Fig. 3). Connectives exist between the dorsal brain cells, and the ventral nerve cord cells, and nerve-like processes are seen in the epidermis and in close association with muscle developing in the pharynx, the setal sacs and the body wall, including both circular and longitudinal musculature. Thickenings of either the nerve or the muscle plasmalemmae are sometimes seen as the nerve runs along beside the muscle cell (Fig. 4). These might represent synaptic areas, being highly comparable to neuromuscular junctions reported in larvae of Arenicola cristata (Marsden and Lacalli, 1978) and Harmothoë imbricata (Holborow, 1971). No nervous connections with ciliated cells of the trochal bands were found, unlike Harmothoë imbricata larvae, in which neurociliary synapses have been described (Holborow, 1971; Holborow, Laverack, and Barber, 1969).

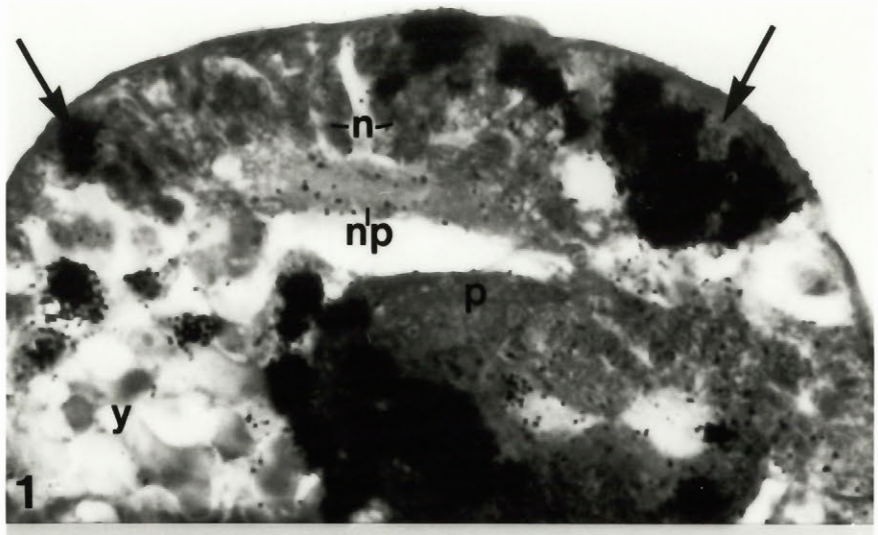
A second type of cell, elongated and resembling a fibrocyte, with darkly stained cytoplasm, appears and begins to separate the cells of the developing nervous system from other non-neural cell groups such as muscle, epidermis and gut (Fig. 5).

Nervous System Development in Hatched Larvae

Growth of the nervous system after hatching has occurred involves continous addition to the elements formed during the red pigment band stage. Fibrocyte-like cells gradually ensheathe the ganglionic clumps of cells, the distinct connective tissue capsule of the brain being incomplete dorsally until the larvae reach 10 to 15 setigers in length. In addition, other darkly staining cells, presumed to be differentiating glia, become interspersed as satellite cells among the neurons. Undifferentiated cells remain scattered throughout the brain, and some continue to label with tritiated thymidine until brain growth is completed (See Easdown, Marsden, Paradis, Bell and Jost, in preparation). Nerves running out to the various head appendages, such as palps, frontal antennae, eyes, and tentacular cirri, appear as these structures do. An increase in the number of differentiated neurons is observed as the larvae grow, and cells with a wide variety of inclusions, both dense granules and lucent vesicles, resembling those seen in adult neurons, appear (Fig. 6). Neuropil fibres, 0.2 to 1 micron in diameter, contain a similar assortment of granules and vesicles. Specialized neurons, such as d cells, which are possible photoreceptor components (see Dhainaut-Courtois, 1965), appear in 3-setiger larvae, and cells of the corpora pedunculata, possible sensory association centres (see Bullock and Horridge, 1965), are first seen in animals of 15 setigers. Paraldehyde fuchsin-positive cells are first distinguishable in the hindbrain in larvae of 25 to 30 segments. These presumptive neurosecretory cells

Figure 1. Longitudinal paraffin section of a late red pigment band stage larva. Note the pharynx (p) and yolky reserves (y) in the body cavity. Some neuropil (np) is apparent in the head, surrounded by undifferentiated cells of the developing brain (n). Lateral clumps of undifferentiated cells (arrows) are labelled with tritiated thymidine. 1000X.

Figure 2. Electron micrograph of undifferentiated cells within the brain, and adjacent neuropil tissue (np). The cells are rich in glycogen, and the nerve processes contain various dense granules. Late red pigment band stage. 7,668X.


n
np
p
y
1

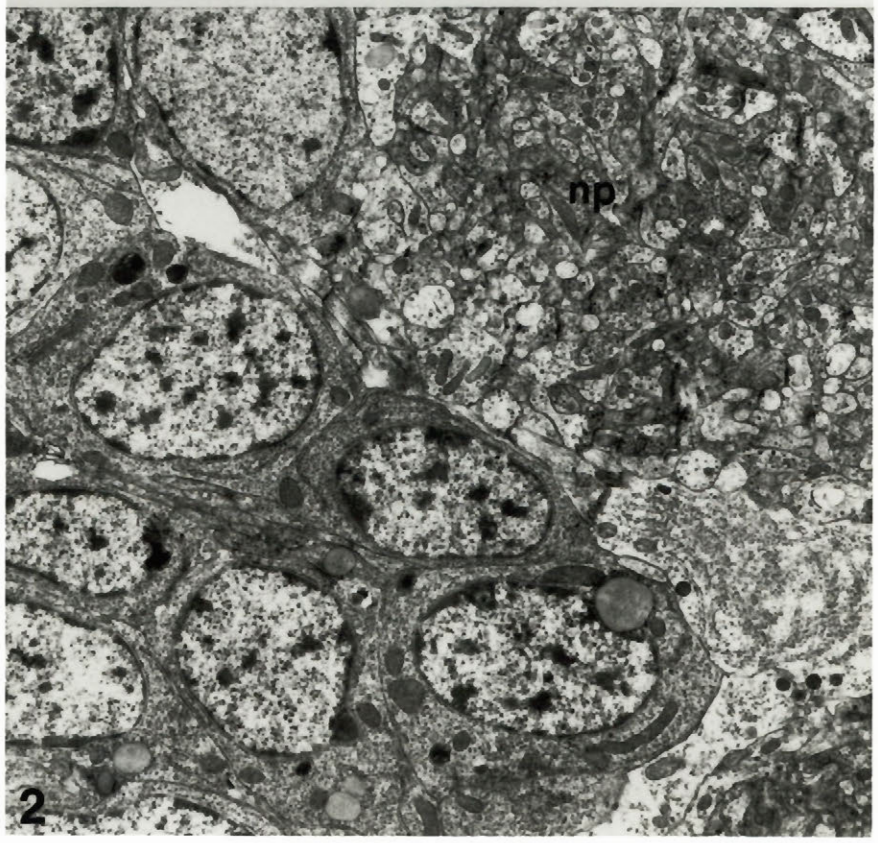
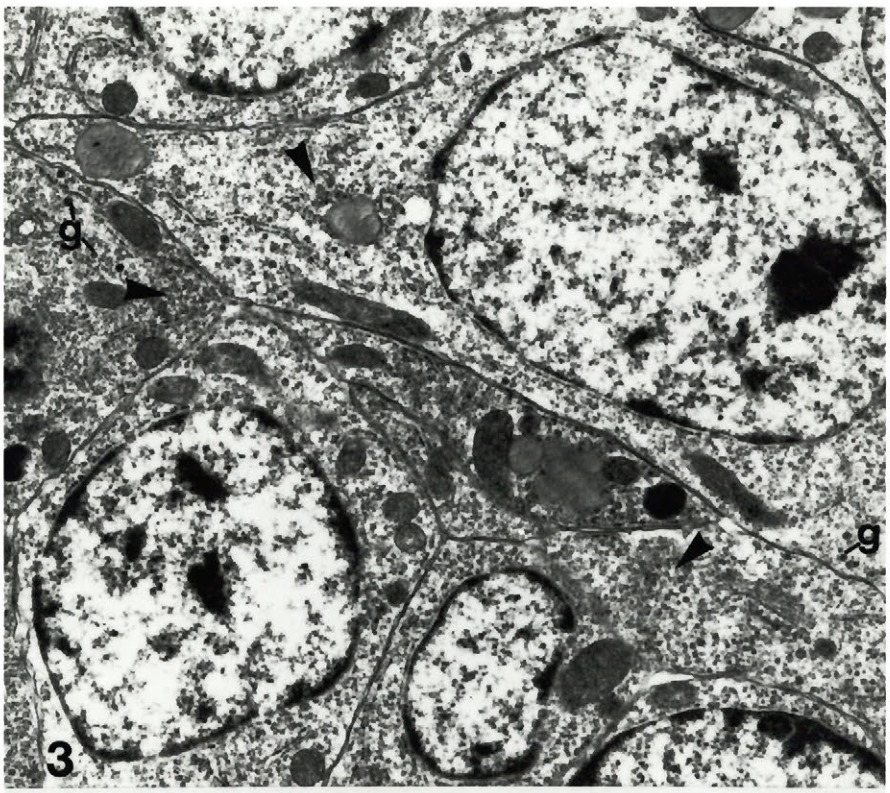

np
2

Figure 3. Differentiating neurons, one with a process. The cells seen here contain a few dense granules (g), as well as copious glycogen stores (arrows) throughout the cytoplasm. Late red pigment band larva. 12,888X.

Figure 4. Possible neuro-muscular junction in parapodium. Nerve processes are full of vesicles (v) and some dense granules (g). Thickenings of the neuronal and muscle cell membranes are apparent (arrows). 37, 656X.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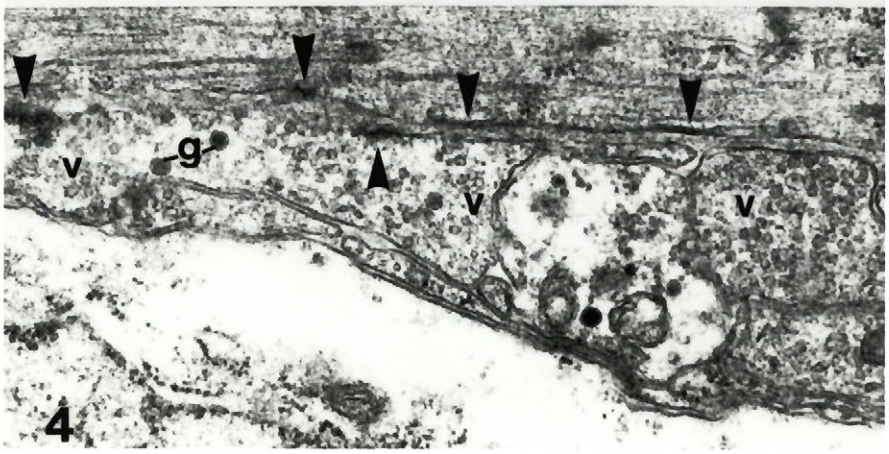
3


v
g
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v
4

Figure 5. Densely stained cells (arrows) separate cells of the brain (n) from cells of the epidermis (e). 4,536X.

Figure 6. Differentiated neurons of later larva (28-setiger). Note variety of dense granules (g) that occur. Glycogen stores are somewhat reduced compared to those in neurons of younger larvae (Fig. 3). 7,668X.


e
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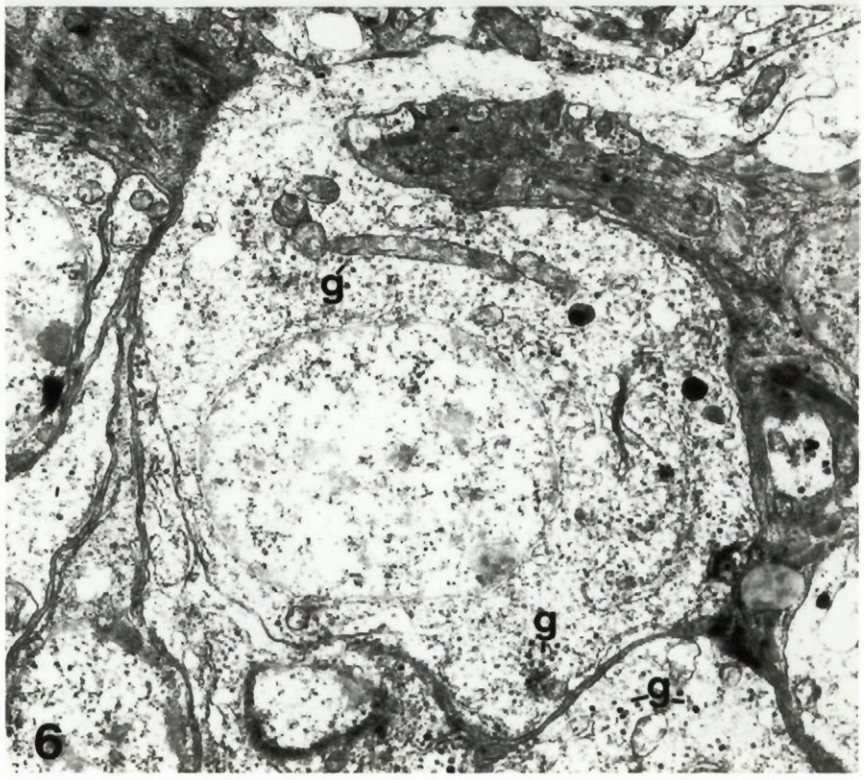
e
n
n
n
e
5


g
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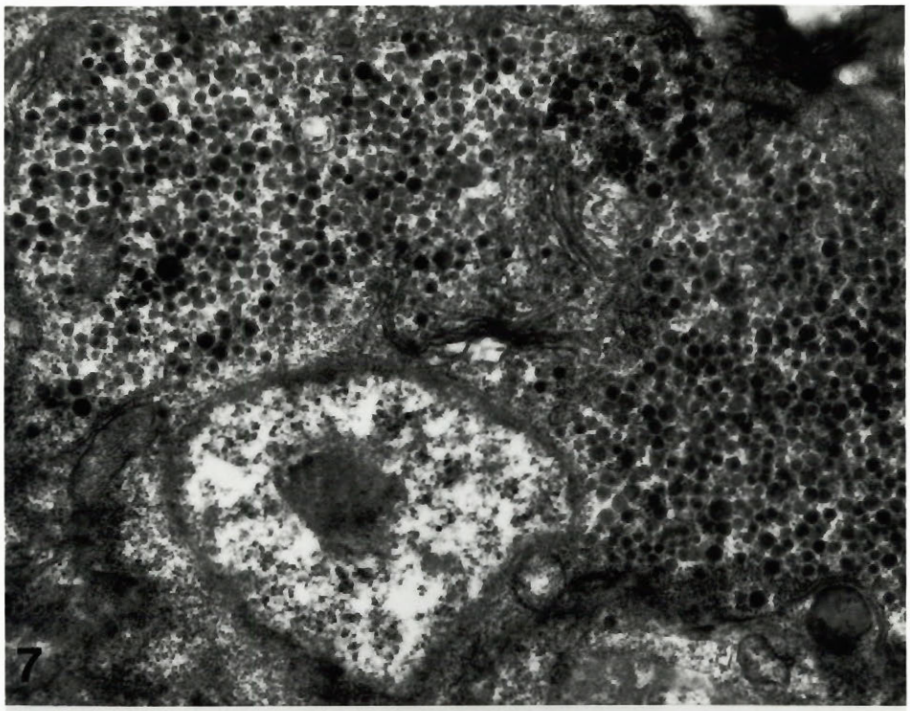
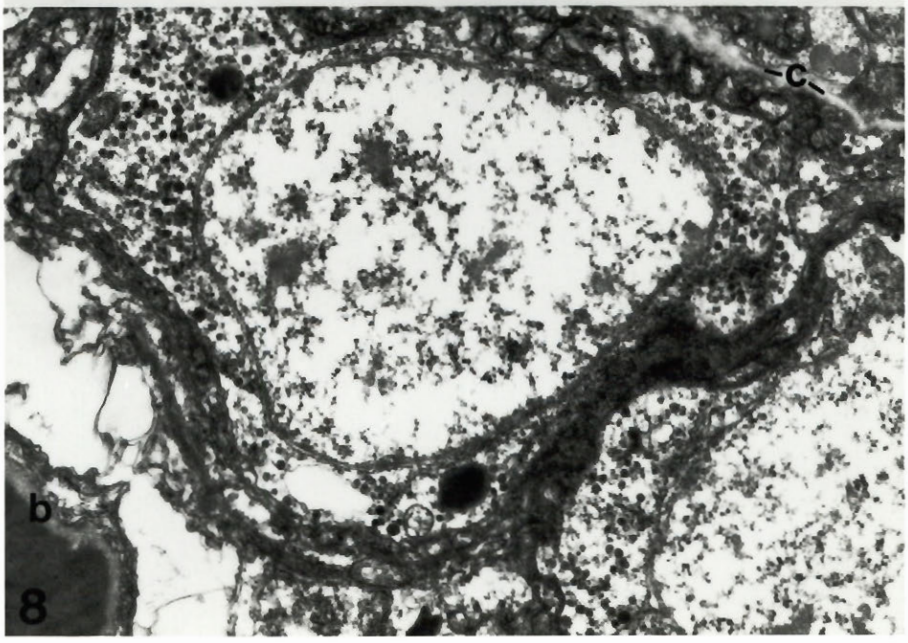
g
6

Figure 7. Presumptive neurosecretory r cell in 28-setiger larva. These cells, full of electron dense elementary granules (up to 2000 A in diameter) are among the first paraldehyde fuchsin-positive cells to appear in the brain. 17,900X.

Figure 8. Early cells in the infracerebral gland of a 21-setiger larva. The granules in these cells range up to 1200 A in diameter. The collagenous capsule (c) of the brain, and the dorsal blood vessel (b) are indicated. 12,888X.


7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b
8

derive from the same precursors as all the other neurons, and include the ventral cells of nucleus 22 and the dorsal r cells of nucleus 20 (see Chapters 3 and 4 of this thesis) (Fig. 7). Both groups of cells contain numerous electron dense granules, ranging up to 2000 A in diameter in the r cells, and up to 1500 A in the cells of nucleus 22. An infracerebral gland was first noted in worms of about 20 segments. Within this gland cells containing dense granules, up to 1200 A in diameter, were located (Fig. 8). These cells resemble the type II (Dhainaut-Courtois, 1968b) or b cells (Baskin, 1974) of the adult infracerebral gland.

DISCUSSION

The Nereis virens larvae cultured in the course of this study showed a pattern of development virtually identical to that reported by Snow (1972) in her study of the natural history of a second New Brunswick population of these worms. Growth rates after hatching were somewhat accelerated in the present study, undoubtedly attributable to nutritional differences in the culturing procedures, since Snow did not feed her larvae. However, Bass and Brafield (1972) have described larval growth in a Thames population of N. virens and certain differences between that population and this are apparent. A gelatinous envelope was not always present in the Thames larvae, and those animals hatched in as little as 28-30 hours post-fertilization as opposed to 7 days post-fertilization in this group. Cilia and setae developed after hatching, and no red pigment band was mentioned in association with the prototrochal cilia. A planktonic phase did occur in the Thames larvae, but apparently did not exhibit a phototactic response. Finally, the Thames animals spent approximately ten days in a 3-setiger nectochaete

phase before beginning to feed, add segments and grow rapidly. No such arrest in development was noted in the larvae observed here. It might also be mentioned that N. virens from the Thames population spawn as heteronereids, while breeding N. virens from New Brunswick are atokous. Thus several differences exist between the two populations both throughout early development, and in adult life as well.

Growth of the larvae in the present population was seen to be rapid and continuous. The larvae achieve adult form progressively, with no abrupt metamorphic changes occurring between these two phases of the life cycle. Nervous system development parallels this progressive manner of somatic growth. Undifferentiated epidermal cells (lateral clumps) possibly give rise to a stock of nervous system cells, some of which continue to divide, some of which differentiate into neurons. The net result is a gradual increase in the size of the nervous system as the larva grows. N. virens larvae are not endowed with specialized larval structures, except the cilia, which do not appear to be under nervous system control, and therefore no radical alterations are required in the nervous system as the change from larva to adult occurs. Certain other species which have been studied manifest gross anatomical changes, such as a decrease in the diameter of the head and a shift in the position of the nuchal organ (Armandia brevis, Hermans, 1978), asymmetrical abdominal growth, loss of mucous gland cells and larval eyespots (Spirorbis moerchi, Sp. spirillum, Sp. vitreus, Potswald, 1978), in the course of settling and metamorphosis of the larval stages. Although morphological changes in the nervous system during the development of these worms have not been looked for, some rearrangements of their nervous systems with metamorphosis might

be predicted. Nereis virens, on the other hand, may serve as a model of nervous system development in a non-metamorphosing Annelid.

The polychaete Arenicola cristata is the only other species to date for which an ultrastructural description of the complete larval nervous system exists (Marsden and Lacalli, 1978), but only one stage in development has been described and any changes which might be associated with its metamorphosis are consequently unknown. However, certain similarities occur between A. cristata and N. virens including the existence of lateral clumps of epidermal cells which seem to give rise to the early nervous system cells, as well as the nature of these cells. Both "lucent" and "dense" cells have been noted in the larval nervous system of Arenicola, the lucent cells resembling the first differentiated N. virens neurons, and the dense cells resembling the fibrocytic or glial cells described here.

The d cells, which are among the earliest recognizable neurons in Nereis virens, appear first in 3-setiger hatchlings . These cells may have a photoreceptor function (Dhainaut-Courtois, 1965), and although their connections have never been elaborated, it might be speculated that they are responsible for the phototactic response noted in the hatchlings. These cells possess remnants of a ciliary apparatus, and since the only ciliated cells in the larvae are in the trochal bands, it might further be speculated that the d cells take their origin from prototrochal cells. The fact that these d cells differentiate before any neurosecretory cells are apparent would seem finally to discount the possibility that they arise as certain secretory neurons empty of their contents (Scharrer, 1936; Takeuchi, 1965).

In considering the overall pattern of development of the Nereis virens brain, it is of interest that the anterior and middle portions of this ganglion are essentially the first to appear in a differentiated form. The ganglionic nuclei of fore- and mid-brain seem, in the adults, to be associated with 13 of the 15 nerves that connect with the brain. These nerves, both sensory and motor in function, serve primarily the head and its specialized appendages. The hindbrain, including nuclei 20 and 22, which contains a large proportion of the total number of presumptive neurosecretory cells in the adult brain (see Chapter 3 of this thesis), is the last to finish differentiating recognizable nuclei. Thus despite the importance of neurosecretion in older specimens, it may be that the function of the larval brain is strictly sensory-motor, and that no neurohumoral controls are exerted by the brain during initial development. This may not be true of all polychaetes, since Korn (1958, 1960) has identified, with the light microscope, cells he believes to be neurosecretory in the trochophore larvae of Pectinaria sp., Nephthys sp. and Harmothoë sp.

The range of normal behaviours observed in N. virens confirms that functional neuromuscular connections have been made by the time that hatching occurs. Acetylcholine, serotonin and glutamic acid all may be active neurotransmitters in this system. It is not clear at which level these substances are acting, since the effects of all three drugs was roughly similar. Results are consistent with the possibility that acetylcholine, which produces the fastest reaction, acts directly on the muscles, while serotonin and glutamic acid act via the central nervous system, or modulate transmission at the neuromuscular junction. Control of the gut, in particular a relaxation response, may be cholinergic as well, since the muscarinic blocker atropine produces strong gut contractions.

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CHAPTER 7

DISCUSSION, CONCLUSIONS AND SUMMARY

DISCUSSION

In the course of the foregoing thesis the anatomy of the supraoesophageal ganglion of Nereis virens has been described, and is seen to closely resemble that of other previously examined Nereids (Holmgren, 1916; Golding, 1967; Dhainaut-Courtois, 1966, 1968). Cells of possible neurosecretory significance have been located in 17 of the 26 ganglionic nuclei, however, the majority of these cells are to be found in nucleus 20. Nucleus 20 is not of homogeneous composition, as has been previously noted (Golding, 1967) and in the present study four cell types, p,q,r and s cells, were found within this nucleus. These cells have been characterized by both light and electron microscopy, and their relative positions in the nucleus have been described. Histologically two of the four cell types, p and r cells, are comparable to certain neurosecretory cells which have been reported in other Nereid brains (type I and II cells, respectively, in Nereis pelagica, Dhainaut-Courtois, 1966, 1968; and nucleus XIXS (20) of N. diversicolor, Golding, 1967). These p and r cells reach maximum staining intensity with PAF and maximum size and number in older worms, therefore it has been suggested that they perform some secretory function in later stages of the life cycle. Electron microscopic investigation would seem to confirm that p and r cells are neurosecretory, since they are especially rich in the large electron dense elementary granules that are usually associated with secretory cells, and in addition would seem to confirm that these cells maintain secretory activity in adult specimens, since active Golgi bodies and quantities of elementary granules and rough endoplasmic reticulum are observed in the p and r cells of sexually mature animals. The p cells maintain a relatively unchanging appearance throughout the life cycle, so it has been suggested that they

play a trophic role, possibly related to gamete maturation. Such an hormone has been described in Platynereis dumerilii, for example (Müller, 1973). However, there is an obvious decrease in the number of dense granules stored in the r cells immediately after spawning, therefore it has been postulated that these cells in particular may be the source of some hormone which triggers spawning. Indeed it has been shown that injections prepared from the brains of animals which have spawned, are in some cases capable of inducing spawning in gravid recipients. These results support the idea that a spawning hormone exists in the Nereis virens brain. This is the first indication that has been uncovered in regards to such an hormone in the Nereidae. The mode of action of this substance remains undetermined, but it is clear that any humoral control over spawning could be an important means of coordinating the breeding swarms of a given population of worms. Environmental influences such as temperature and photoperiod are known to affect neuroendocrine activity in various other polychaetes (Durchon and Porchet, 1971; Hauenschild, 1955, 1960, 1961) so it would not seem unreasonable to assume that they might also operate in this case. Nereis virens has been noted to spawn in the laboratory in response to increased water temperature, so it is possible that some such external cue may trigger the release of spawning hormone from the brain, resulting in the spawning of many gravid adults at one time. This may be true in other Nereids as well, since Dales (1950) has reported that N. diversicolor, which also spawns in the spring, may do so in response to heightened temperatures.

Previous investigations have concentrated, to a large degree, upon hormones which have been described in the juvenile Nereid brain (see Introduction). A neuronal source of "juvenile hormone" has not been

definitely localized in those studies, although the type I neurons (equivalent to p cells) have been put forth as candidates (Dhainaut-Courtois, 1968). This possibility is somewhat discounted by the present study, since p cells (type I cells) of N. virens are among the last neurons to differentiate in nucleus 20, and since they occur throughout the life cycle, even into sexual maturity. However, these cells, and the r cells as well, possess the ultrastructure of secretory neurons from the time of their first appearance in the brain, as well as in adult brains. It is therefore suggested that, rather than being a source of strictly "juvenile" or strictly "adult" hormones, these cells are functional throughout the life span of N. virens, from juvenile stages through to spawning, possibly changing their product(s) with age. There is some slight evidence for this latter conjecture, since an inexplicable increase in the size of elementary granules in these cells was noted with age, and since the staining characteristics, in particular with PAF, likewise changed with age. Lack of or reduced staining with PAF in no way implies that the cells are not of a secretory nature. Bag cells of Aplysia californica, for example, which are proven to be neurosecretory (ie. Toevs and Brackenbury, 1969; Pinsker and Dudek, 1977; etc.) do not stain with PAF (see Appendix I). PAF, however, appears largely to stain acidic groups, therefore a change in PAF staining with age, such as was observed in the p and r cells of N. virens, may be related to alterations in the chemical composition of cell contents. Although there is no precedent for the assumption that p and r cells may change their respective outputs with age, it is not an impossibility, and would indeed seem to be an economical manner in which to utilize those neurosecretory cells which are available in the brain. Neurons which are presumed non-secretory far outnumber those which

may be secretory in the Nereid brain (see Dhainaut-Courtois, 1966, 1968; Golding, 1967).

The development of the nervous system including neurosecretory cells, and the behaviour of Nereis virens larvae, have here been discussed for the first time. These larvae are not specialized in any particular way, nor do they undergo drastic metamorphosis during development, but instead hatch as "miniature adults", and grow by the progressive addition of segments and appendages, to adult size. Nervous system development occurs in a parallel fashion, with no apparent metamorphic changes. Cells of epidermal origin form the ganglionic rudiments, then some of these cells continue proliferation while some differentiate, resulting in a gradual increase in the number of recognizable nervous system elements concomitant with somatic growth. The nervous system has certainly made functional connections by the time the larvae have hatched at seven days post-fertilization, since the animals can swim, crawl, feed and respond to various neurotransmitters at that time. For a period of a day or less, immediately after hatching, the larvae are positively phototactic. This pelagic phase possibly serves a purpose in dispersing the animals to appropriate locations within their home mudflats, but since the flats may be quite limited in expanse in some locales, it need not be long-lasting.

It is most interesting that neurons with the ultrastructure of secretory cells are among the last to develop within the larval brain. Therefore, in regards to N. virens, it has been suggested that the brain may play no secretory role in larval life, and even that there may be no humoral requirements for larval growth. Such controls may only come into effect in post-larval/juvenile life. It might even be speculated

that juvenile hormone(s) might interfere with proper larval development, since it has been reported that insect juvenile hormone causes abnormalities in embryogenesis in, for example, the desert locust Schistocerca gregaria (Injeyan, et al, 1979).

It is also interesting that the neurosecretory cells that were found in the early brain appear to have the same antecedents as all the other neurons. Only one type of undifferentiated precursor cell was seen, apparently giving rise to all nervous system components. This would seem to establish a direct ancestral relationship between secretory and non-secretory neurons, in opposition to the theory of R.B. Clark (1956) that neurosecretory cells are primarily secretory in nature, being derived from epidermal secretory cells, and only secondarily acquiring the characteristics of neurons. Based on the present findings, it is postulated that the neurons take on their adult characteristics, either secretory or non-secretory, only after their processes have grown out to their appropriate targets, and/or after presynaptic cells have contacted them. This sort of notion is supported by, for example, the experiments of Goodman, et al (1979), which indicate that identified dorsal unpaired median cells of Schistocerca nitens differentiate morphologically and biochemically only after their axons have reached their post-synaptic targets.

CONCLUSIONS

Despite morphological examination of the brain of Nereis virens throughout the life cycle, it has been impossible to localize a single source of the "juvenile hormone" that plays such a prominent role in growth and maturation in this family. Production of such an hormone might of course be localized in some of the other neurosecretory cells

of the supraoesophageal ganglion which have not been examined in detail here. However, this study of nucleus 20 in particular, raises the distinct possibility that hormonal controls in this species, and by generalization in other Nereids, may be much more varied than initially suspected, and may exist at all stages in the life cycle except during larval develoment.

SUMMARY

1. The microanatomy of the brain of Nereis virens has been examined by both light and electron microscopy, and has been found to closely resemble that of certain other Nereids.

2. One specific ganglionic nucleus, nucleus 20, has been studied in detail. Four cell types were identified in this nucleus, of which two types, p and r cells, are apparently neurosecretory.

3. P and r cells have the ultrastructure of secretory cells from the time they first appear in the brain, but reach maximum staining intensity with paraldehyde fuchsin, and maximum size and number in adult worms. It has therefore been suggested that they may be functional neurosecretory cells throughout the life history of Nereis virens, and in particular, that the p cells may play a trophic role in development and maturation, while r cells may secrete an hormone associated specifically with spawning.

4. Experiments on gravid animals indicate that an hitherto undescribed hormone inducing spawning may exist in Nereis virens. Extracts prepared from the brains of animals which had spawned were found capable, in some cases, of causing spawning in recipient worms when injected.

5. The development of the nervous system in laboratory cultured larvae was examined. All the cells of the brain seem to arise from lateral clumps of epidermal cells in the head. Some cells have begun to differentiate processes and functional connections are being made by the time the larvae hatch as 3-setigers at seven days post-fertilization. Other cells within the nervous system continue to divide at the same time, with the net result being a gradual increase in the number of neurons, paralleling bodily growth. No abrupt somatic metamorphosis

occurs, nor are there any abrupt nervous system changes as the larvae grow.

6. After hatching the larvae swim, crawl and feed, and respond to the neuropharmacological agents acetylcholine, serotonin and glutamic acid. It has been suggested that acetylcholine acts directly at the neuromuscular junction in these animals, while serotonin and glutamic acid either act via the central nervous system or modulate transmission at the neuromuscular level.

7. Neurosecretory cells are among the last cells to differentiate, appearing only in older larvae of 25 to 30 setigers. Therefore it was suggested that there is no endocrine activity by the brain until post-larval life, but that the primary function of the larval brain is strictly sensory-motor.

8. In effect, the possibility has been raised that neurosecretory controls in Nereis virens may extend through juvenile life into adulthood, but may not exist during larval development.

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APPENDIX I

DIFFERENTIAL STAINING OF NEUROSECRETORY CELLS IN *Nereis virens* WITH PARALDEYHYDE FUCHSIN

ABSTRACT

The reactions of two neurosecretory systems, the hindbrain of the Annelid Nereis virens, and the bag cells of the Mollusc Aplysia californica, to paraldehyde fuchsin (PAF) and other histological stains, have been examined. PAF used at a low pH (1.3 to 1.6) selectively stains only those neurons rich in cystine/cysteine, while a higher pH solution (3.0 to 3.3) stains additional cells which contain other reactive acid groups, but not cystine or cysteine. Cells which are not especially acidic do not stain with PAF, even if they are rich in aldehydes, and aldehyde blockage does not prevent PAF staining. PAF, as utilized here, seems to be a very useful indicator as to exactly which acid residues, if any, are present in the neurosecretory cells.

INTRODUCTION

Paraldehyde fuchsin (PAF) has been used to demonstrate neurosecretory material in many species, both vertebrate and invertebrate, since it was first applied, with positive results to the frog pituitary (Dawson, 1953). PAF may have some of the properties of a Schiff base (Bangle, 1954), and in some cases may react with aldehydes (Scott and Clayton, 1953), however, it appears to depend largely on the presence of tissue anions for its staining reaction (Konecny and Pliczka, 1958; Buehner, et al, 1979). As has been previously reported, staining results are often affected by the method of stain preparation (ie. Gomori, 1950; Gabe, 1953; Rosa, 1953), by the quality of reagents used to make the stain, by the oxidation procedure used, etc. (Meola, 1970; Mowry, 1978).

In the foregoing thesis, PAF was applied to the brain of the polychaete Nereis virens, and two populations of PAF positive neurons, p and r cells, were distinguished by the use of two different PAF techniques (see Chapter 3). Both p and r cells stain when using the Cameron and Steele (1959) method (PAF prepared according to Gabe, 1953), while only p cells stain by the Meola (1970) method (PAF prepared according to Rosa, 1953). Further histochemical analysis of the brain of Nereis virens, and the bag cells of Aplysia californica, which form a well-documented neurosecretory system (Toevs and Brackenbury, 1969; Pinsker and Dudek, 1977; etc.), has been undertaken in an effort to explain the differential staining of p and r cells that was achieved.

MATERIALS AND METHODS

Adult Nereis virens were obtained from Marine Research Associates, Ltd., Deer Island, New Brunswick. Prostomia were fixed 24 hours in Bouin's fluid and paraffin embedded. Five micron sections were stained according to the references listed in Table 1. In addition to using two PAFs, prepared, using identical reagents, by the methods originally described (Cameron and Steele, 1959; Meola, 1970), the pH of the staining solutions was varied (Table 2), periodic acid was tested as an oxidant instead of acidified permanganate, controls were stained in the absence of oxidation, and aniline was applied in conjunction with PAF, as an aldehyde blocker.

Adult Aplysia californica were obtained from Pacific Bio-Marine Laboratories, Inc., Venice, California. Abdominal ganglia bearing the bag cell clusters were dissected out and fixed 24 hours in Bouin's fluid. Five micron paraffin sections were stained concurrently with sections of Nereis virens brains.

RESULTS

Results obtained with the various stains are summarized in Table 2. In the Nereis virens hindbrain three groups of cells give interesting results. The type III cells of the nuchal nucleus (nucleus 18) (Dhainaut-Courtois, 1966) colour brilliantly with eosin in hematoxylin-eosin stained slides (Fig. 1), and are strongly Schiff-positive, indicating, respectively, the presence of basic substances and neutral mucopolysaccharides with these cells. They never stain with PAF, but rather take up the light green counterstain. By virtue of their high affinity for acidic stains (ie. eosin and light green), they will be referred to as acidophils. On the other hand, the p and r cells of nucleus 20 (see Chapters 3 and 4 of this thesis, for description of these cells), react weakly with Schiff reagent and Alcian blue,

Table 1. Stains applied to the Nereis virens brain, and the Aplysia californica abdominal ganglion.

Stains	References
Delafield's hematoxylin and 0.1% eosin Y in 70% ethanol	Humason, 1967.
0.1% toluidine blue in 30% ethanol	Pearse, 1961.
0.5% Alcian blue in 3% acetic acid	Carleton, 1967.
periodic acid-Schiff (PAS)	Thompson, 1966.
Victoria blue following thiosulphation	Victoria blue 4R, as prepared by Dogra and Tandan, 1964, used with the thiosulphation procedure of Castino and Bussolati, 1974 (Appendix II of this thesis).
paraldehyde fuchsin (PAF) with Halmi's trichrome counterstain, acidified permanganate oxidation	Cameron and Steele, 1959. Meola, 1970. Halmi, 1952.
10% aniline in acetic acid before PAF staining	Pearse, 1961.
PAF after five minutes oxidation in 0.5% periodic acid	PAF preparation and staining as in Cameron and Steele, 1959, and Meola, 1970.

Table 2. Summary of staining results

	cell types				significance of positive results
	acidophils (nucleus 18)	p cells (nucleus 20)	r cells (nucleus 20)	bag cells	
hematoxylin eosin	eosin +++	hematoxylin +	hematoxylin +++	hematoxylin +	Eosin stains basic materials, hematoxylin stains acidic materials.
Alcian blue	-	weak +	weak +	weak +	Alcian blue stains acid groups especially from mucopolysaccharides.
toluidine blue	background	background	dark bluish-purple (weak β meta chromasia?)	background	Toluidine blue stains acid groups.
PAS	+++	weak +	weak +	weak +	PAS stains mucopolysaccharides, especially neutral polysaccharides.
Victoria blue after thiosulphation	-	+++	-	-	Thiosulphation makes cystine/cysteine residues stainable.

Table 2 (cont'd)

	acidophils	p cells	r cells	bag cells	significance
PAF, with Halmi's counterstain, acidified permanganate oxidation.					
Meola, original pH = 1.5	Light green +++	PAF +++	background PAF	background PAF	Light green stains basic groups, PAF seems to stain only strongly acid groups, such as cysteic acid from cystine/ cysteine, or carboxylic acids. See text for discussion.
Meola, at pH = 3.2	Light green +++	PAF +++	PAF +++	background PAF	
Cameron and Steele at pH = 1.5	Light green +++	PAF +++	background PAF	background PAF	
Cameron and Steele, original pH = 3.2	Light green +++	PAF +++	PAF +++	background PAF	
PAF controls without oxidation, any pH	PAF- (light green +++)	PAF- (light green +)	PAF- (light green +)	PAF- (light green +)	Rules out the possibility that free sulfonic acid groups from sulphated muco-polysaccharides are responsible for positive reaction with PAF after oxidation.

Table 2 (cont'd)

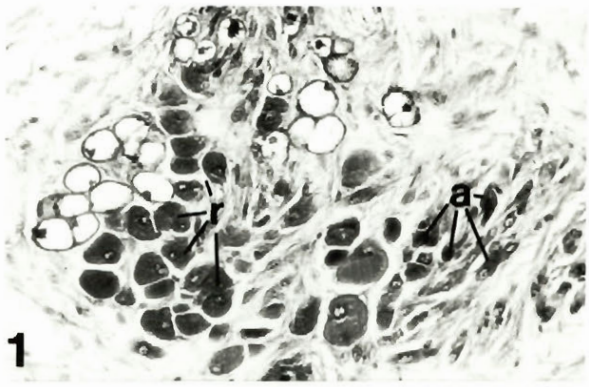
	acidophils	p cells	r cells	bag cells	significance
PAF after aniline treatment, permanganate oxidation					Rules out the possibility that PAF staining of p and r cells is due to aldehydes.
PAF, pH = 1.5	PAF-	PAF +++	background PAF	not tested	
PAF, pH = 3.2	PAF-	PAF +++	PAF +++	not tested	
PAF after periodic acid oxidation, any pH	PAF- (light green +++)	PAF- (light green +)	PAF- (light green +)	PAF- (light green +)	Rules out the possibility that lack of staining of acidophils and bag cells is due to the destruction of aldehydes by acidified permanganate. Also indicates that positive staining of p and r cells is not due to is aldehydes.

indicating the presence, in low quantities only, of neutral and acidic mucopolysaccharides in these cells. The p cells react positively in the thiosulphation-Victoria blue method used for the demonstration of cystine/cysteine. The r cells stain with unusual intensity with hematoxylin (Fig. 1), and exhibit a deep bluish-purple staining with toluidine blue that borders on β-metachromasia. R cells therefore appear to be rich in acid groups, probably from protein or glycoprotein, since few mucopolysaccharides seem to be present. When PAF, prepared and applied according to either Meola or Cameron and Steele, is used at a pH of 1.3 to 1.6, only p cells stain (Fig. 2). This pH is close to the pK of cysteic acid, the oxidation product of cystine/cysteine, so it is likely that such residues are responsible for the PAF-positive reaction of the p cells. At a pH above 3.0 all preparations of PAF stain r cells as well as p cells (Fig. 3). This pH approximates the pK values of the carboxyl groups of many of the amino acids, making it seem probable that the acidic groups within the r cells are largely carboxyls. No PAF staining was observed in the absence of oxidation, eliminating the possibility that the positive reactions observed were due to free sulfur acid groups on sulphated mucopolysaccharides, nor was PAF staining of p and r cells hindered by the application of an aldehyde blocking agent (aniline).

Bag cells react only weakly with Schiff reagent, and Alcian blue, and negatively with Victoria blue after thiosulphation, indicating that few mucopolysaccharides are present, and that the amino acids cystine and cysteine are absent from these cells. Bag cells stain at normal intensity with hematoxylin, and show no metachromasia with toluidine blue, therefore likely do not contain any abundance of other acidic compounds. Bag cells do not stain with any of the PAF solutions (Fig. 4).

Figure 1. Hindbrain of adult Nereis virens stained with hematoxylin and eosin. The r cells of nucleus 20 stain with extra intensity with hematoxylin, while the acidophils (a) of nucleus 18 stain strongly with eosin. 250x.

Figure 2. Hindbrain of N. virens stained with PAF (Meola, 1970) at a pH of 1.5. Only the p cells are PAF-positive. The r cells stain at background intensity with PAF, and the nucleus 18 acidophils stain with light green. 250x.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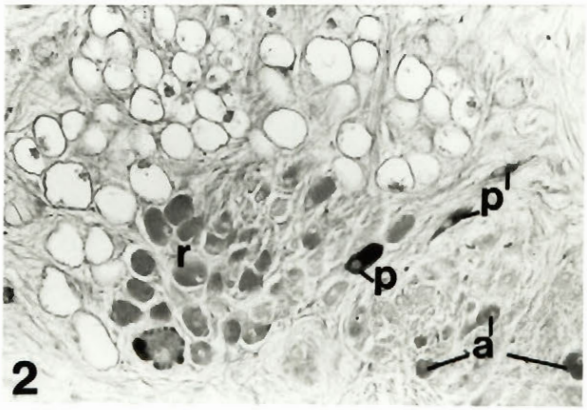
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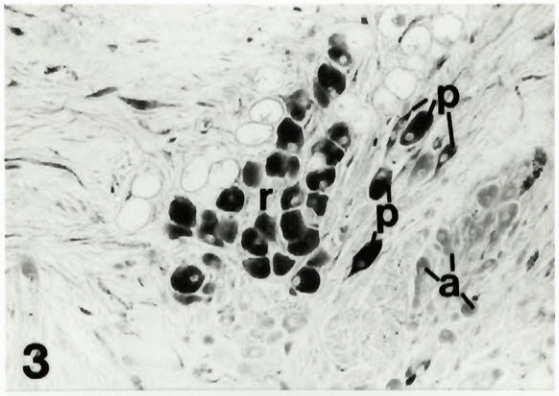
Figure 3. Hindbrain of N. virens stained with PAF (Cameron and Steele,1959) at a pH of 3.2. Both p and r cells are PAF-positive. 250x.

Figure 4. Bag cells of Aplysia californica stained with PAF (Cameron and Steele, 1959) at a pH of 3.2. These cells stain only at background intensity with PAF. Compare with Fig. 3. 250x.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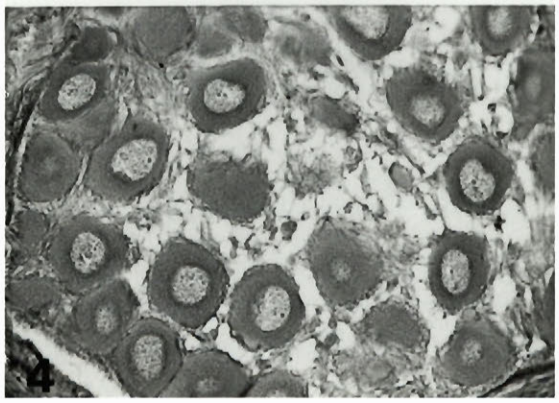
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No PAF staining occurs after periodic acid oxidation, indicating that the positive reactions of p and r cells after permanganate oxidation are not attributable to aldehydes, and that the negative reactions of the acidophils and the bag cells in the same case are not due to the possible destruction of aldehydes by the permanganate (Lillie, 1951).

DISCUSSION

As observed here, PAF staining can be highly pH dependent. PAF at a pH of 1.3 to 1.6 stains only those cells, such as p cells, which are rich in cystine/cysteine. Additional cells, such as the r cells, which contain other acid groups, but not cystine or cysteine, will stain at a higher pH, presumably as their contents become ionized. Aldehyde blockage does not affect the staining of either cell type. Furthermore, the acidophils of nucleus 18, and the bag cells of Aplysia californica, which contain neither cystine/cysteine nor other acid groups, cannot be visualized by any of the PAF's used, despite the fact that the former are Schiff-positive.

It appears, therefore, that differential staining of the p and r cells by PAF is due to the reaction of this stain with different acid groupings contained within these cells - cysteic acid, from cystine/cysteine, in the p cells, and carboxylic acids, presumably from other amino acids, in the r cells. PAF, as utilized here, indeed seems sufficiently pH sensitive to warrant the suggestion that it may of use as quite a specific indicator of which acids are present. As a technique it is much less harsh on sections and is shorter than certain other methods which have been used to demonstrate, for example, cystine/cysteine in neurosecretory and other cells (Adams and Sloper,

1956; Dogra and Tandan, 1964; Castino and Bussolati, 1974).

It should, of course, be pointed out that neurons which are PAF-negative should not be concluded to be non-secretory, but only low in cystine/cysteine and other acidic compounds. Bag cells, for example, although proven to be neurosecretory, do not stain at all with PAF.

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APPENDIX II

VICTORIA BLUE AS A SUBSTITUTE FOR ALCIAN BLUE IN THE THIOSULPHATION PROCEDURE OF CASTINO AND BUSSOLATI (1974)

ABSTRACT

The thiosulphation procedure of Castino and Bussolati (1974), for the demonstration of cystine/cysteine, was found to give non-specific results in the nervous system of the polychaete Nereis virens when Alcian blue was used as the stain to visualize these residues. Ambiguities were overcome by the use of Victoria blue 4R, prepared according to Dogra and Tandan (1964), after thiosulphation, instead of Alcian blue.

INTRODUCTION

The thiosulphation technique of Castino and Bussolati (1974) has been reported specific for the demonstration of protein bound cystine/cysteine residues in histological sections. This particular procedure is much less destructive of tissues than certain other methods which have also been used to localize these amino acids, for example the performic acid-Alcian blue method of Adams and Sloper (1956) and the performic acid-Victoria blue method of Dogra and Tandan (1964). However, when Alcian blue is used as a stain for cystine/cysteine following thiosulphation, non-specific results may be obtained. For example, neurosecretory r cells in the brain of the Annelid Nereis virens give a positive reaction with that stain after thiosulphation treatment, despite the fact that they do not contain cystine/cysteine. This ambiguity can be overcome by substituting Victoria blue (Dogra and Tandan, 1964) for Alcian blue in this procedure.

MATERIALS AND METHODS

Prostomia of adult Nereis virens were fixed in either Bouin's fluid or 5% formaldehyde in sea water, according to the requirements of the staining methods used below. Material was paraffin embedded and sectioned at five microns. Formaldehyde fixed sections were stained with Victoria blue following performic acid oxidation (Dogra and Tandan, 1964). Bouin's fixed material was stained 30 minutes with 0.1% Alcian blue (Eastman Kodak Co., lot # C7B) in 3% acetic acid, following thiosulphation (Castino and Bussolati, 1974) or overnight with Victoria blue 4R (Dogra and Tandan, 1964), after thiosulphation.

Victoria blue is prepared by bringing to a boil 200 mls of distilled water containing 0.5 g of dextrin, 2 g of Victoria blue 4R

(Hartman-Leddon Co., C.I. # 690) and 4 g of resorcin. Twenty-five mls of 29% ferric chloride are added, and boiling continued for 3 minutes. The solution is cooled, and the resultant precipitate filtered out and oven dried. A working stain is made by dissolving all this precipitate in 400 mls of 70% ethanol, and adding 4 mls of concentrated HCl and 6 g of phenol. This stain keeps indefinitely.

RESULTS

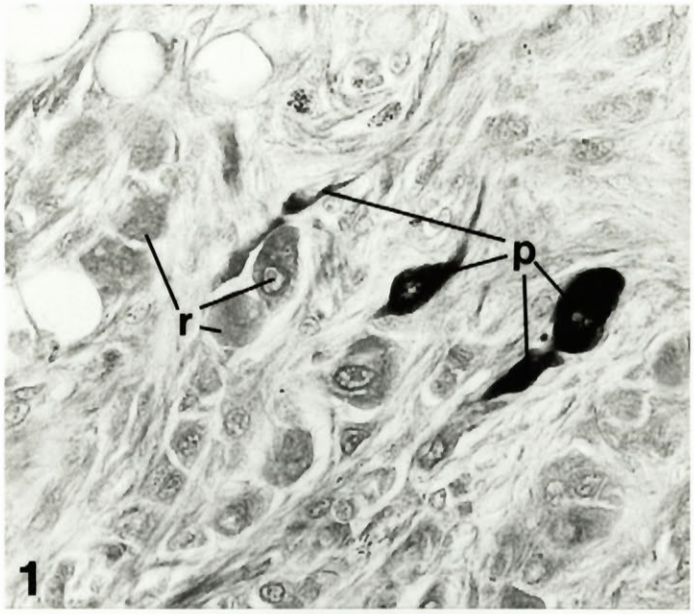
Two putative neurosecretory cell types, p and r cells, have been previously identified in the hindbrain of N. virens (see Chapters 3 and 4, this thesis). In performic acid treated brains p cells stain positively, r cells negatively with Victoria blue, indicating the presence of cysteic acid from cystine/cysteine residues, in the p cells only. Thiosulphated sections stained with Victoria blue give identical results (Fig. 1). However both cell types stain positively with Alcian blue following thiosulphation (Fig. 2). Changes in the stain pH do not alter the results, neither do control sections stain in any case.

DISCUSSION

Since Alcian blue alone stained the r cells following thiosulphation treatment, it is probable that a true "thiosulphation" of the cell contents does not occur in this case. R cells have previously been determined to be high in acidic groupings, apparently carboxyls (see Appendix I, this thesis), and it is possibly these radicals which are somehow rendered reactive during the thiosulphation procedure. The equivocal results obtained with Alcian blue may be due to its ability

Figure 1. The hindbrain of Nereis virens stained with Victoria blue after thiosulphation. P cells stain positively, r cells stain only at background intensity. 640X.

Figure 2. A similar section stained with Alcian blue after thiosulphation. Both p and r cells react positively to this stain. 640X.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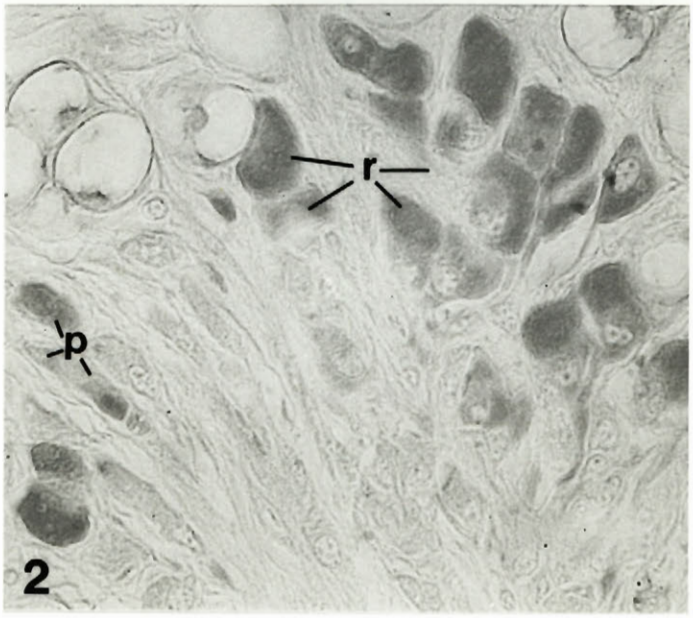
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to bind via amide bonds to certain residues (Spicer, 1960) rather than, or as well as, by simple salt-linkages.

Victoria blue, however, appears to give specific staining of only cystine/cysteine residues, thus is suggested as a replacement for Alcian blue in the thiosulphation technique.

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