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The Development of the Nervous System of *Aurelia aurita* (Scyphozoa, Coelenterata)

by

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**A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of**

DOCTOR OF PHILOSOPHY

in the Department of Biology

**We accept this dissertation as conforming
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ABSTRACT

Aurelia aurita passes through several life cycle stages during its development. Sexual reproduction occurs in the adult jellyfish and results in the free-swimming planula, which develops into the sessile scyphistoma (polyp). The polyp, which reproduces asexually by budding, develops into a strobila which, also asexually, produces free-swimming ephyrae (the initial medusoid stage) through transverse fission.

In the planula, the nervous system consists of ectodermal sensory cells and neurons and their fibres. Anti-FMRFamide antibodies label both sensory and neuronal cells in the anterior region of the planula; the neuronal processes are mostly arranged longitudinally along the anterior/posterior axis, and a few fibres run transversely. Labelled neurons do not appear to make contact with one another in the early, *i.e.* just released, planula and do not have the appearance of a nerve net until the late planula, *i.e.* just prior to metamorphosis.

Metamorphosis of the planula can be induced by exogenously applied thyroxine (10^{-7}M) and iodine (10^{-7}M) and, to a lesser degree, by retinoic acid (10^{-7}M). MgCl_2 (0.13M) and FMRFamide (10^{-7}M), on the other hand, inhibit or reduce, respectively, the induction of metamorphosis. Less than 50% of planulae cut into anterior and posterior halves are undergoing metamorphosis after 10 days, and thyroxine fails to enhance the rate of metamorphosis in those larvae.

In the scyphistoma, the nervous system consists of sensory cells and neurons in the ectoderm and the endoderm. The somata of cells labelled with anti-FMRFamide are located mostly in the oral disc and the tentacles, where they, together with their processes, have the appearance of a nerve net. Nerve fibres are also found on the four muscle bands that extend the length of the scyphistoma from the pedal to the oral disc.

In the developing and adult jellyfish, conventional techniques such as methylene blue staining distinguish between a diffuse nerve net and a giant fibre system. Neuronal subsets are identified by immunohistochemical techniques such as labelling with anti-FMRFamide and a monoclonal antibody generated with ephyral tissue as the immunogen. Anti-FMRFamide labels a subset of neurons of the diffuse nerve net, whereas the monoclonal antibody labels a separate subset of neurons, some of which belong to the giant fibre system, while others do not, and none of which co-label with the anti-FMRFamide antibodies. In contrast to the subset of FMRFamide-positive neurons, which has the appearance of a nerve net in all jellyfish stages, the number of neurons labelled with the monoclonal antibody increases during the development of the jellyfish from a few scattered neurons in the ephyra to an interconnected population of neurons forming a nerve net in the adult. Whereas elements of the diffuse nerve net and the FMRFamide label are present in all life cycle stages, the giant fibre system, which innervates the swimming muscles, and the monoclonal antibody label only occur in the jellyfish stages.

Rhodamine B, which has been used as an indicator of neuronal activity in other phyla, was found to also stain neurons in the jellyfish stages of *Aurelia*. The number of stained neurons was significantly higher in ephyrae treated with FMRFamide or *Artemia* when compared to ephyrae treated with $MgCl_2$ and FM[D-R]Famide.

The data indicate that the nervous system of *Aurelia aurita* is more complex than previously assumed, in that a separate nerve net or subset of neurons is present in the jellyfish whose characteristics are neither solely that of the giant fibre system nor that of the diffuse nerve net. No indications were found for neuronal cell death during the development of the nervous system.

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Table of Contents

<u>ABSTRACT</u>	i
Table of Contents	v
List of Figures	viii
List of Tables	x
List of Abbreviations	xi
Acknowledgments	xiv
Frontispiece	xvi
1 INTRODUCTION	1
The Life Cycle of <i>Aurelia aurita</i>	1
Historical Overview	4
<i>From Obscurity to Mainstream Biology</i>	4
<i>Classification Systems</i>	8
<i>Discovery of the Life Cycle</i>	15
<i>Progress in Neurobiology</i>	20
The Modern Era	51
Objectives	63
2. MATERIALS AND METHODS	66
Animal Maintenance	66
Electron Microscopy	67
<i>Transmission Electron Microscopy</i>	67
<i>Scanning Electron Microscopy</i>	67
Monoclonal Antibodies	67
<i>Production of Monoclonal Antibodies</i>	67

<i>Isotyping</i>	69
<i>Determination of Apparent Molecular Weight of Antigen</i>	69
Immunohistochemistry	70
<i>Immunofluorescence</i>	70
<i>Immunogold Labelling</i>	71
Staining F-Actin with Fluorescent Phallotoxins	72
3. EXPERIMENTAL STUDIES	72
The Planula	72
<i>Introduction</i>	72
<i>Materials and Methods</i>	77
<i>Scanning Electron Microscopy</i>	77
<i>Induction of Metamorphosis</i>	78
<i>Results</i>	79
<i>Gross Morphology and Behaviour</i>	79
<i>Light Microscopical Observations</i>	80
<i>Electron Microscopical Observations</i>	81
<i>Induction of Metamorphosis</i>	86
<i>Discussion</i>	104
The Scyphistoma	112
<i>Introduction</i>	112
<i>Results</i>	114
<i>Gross Morphology</i>	114
<i>Light Microscopical Observations</i>	114
<i>Electron Microscopical Observations</i>	119
<i>Discussion</i>	131

The Free Swimming Jellyfish: Ephyra, Juvenile and Adult	133
<i>Introduction</i>	133
<i>Materials and Methods</i>	143
<i>Methylene Blue Staining</i>	143
<i>Immunofluorescence for Double-Labeling</i>	144
<i>Rhodamine B Staining</i>	144
<i>Effects of Treatments (FMRFamide, Artemia, and Glycine) on the Swimming Beat</i>	145
<i>Results</i>	145
<i>Gross Morphology of the Ephyra</i>	145
<i>Light Microscopical Observations</i>	146
The Muscles.....	146
Methylene Blue Staining.....	146
Anti-FMRFamide-Label.....	147
Monoclonal Antibody Aa Labelling.....	159
Rhodamine B Staining.....	164
Double-Labeling with anti-FMRFamide and mAb Aa.....	171
<i>Electron Microscopical Observations</i>	171
<i>Specificity of Monoclonal Antibody Aa</i>	178
<i>Rhodamine B as an Indicator of Neuronal Activity</i>	183
<i>The Swimming Beat</i>	190
<i>Discussion</i>	195
4. GENERAL DISCUSSION	201
5. LITERATURE CITED	209

List of Figures

Fig. 1 Life Cycle of <i>Aurelia aurita</i>	3
Fig. 2 Planula of <i>Aurelia</i>	81
Fig. 3 FMRFamide-positive neurons in the planula.....	85
Fig. 4 Electron micrographs of ectodermal cells in the planula.....	88
Fig. 5 Electron micrographs of cells in the planula.....	90
Fig. 6 Induction of metamorphosis in <i>Aurelia aurita</i> planulae.....	94
Fig. 7 Effects of treatments (thyroxine, iodine, FMRFamide, and MgCl ₂) on induction of metamorphosis.....	97
Fig. 8 Effects of treatment (thyroxine, iodine, FMRFamide, and MgCl ₂) on attachment of <i>Aurelia aurita</i> planulae.....	100
Fig. 9 Effect of retinoic acid on metamorphosis in planulae.....	103
Fig. 10 Gross morphology of the scyphistoma.....	116
Fig. 11 Muscle bands and associated neuronal processes of the scyphistoma.....	118
Fig. 12 FMRFamide-positive neurons of the scyphistoma.....	121
Fig. 13 The muscle bands and neurons in the strobila.....	123
Fig. 14 Electron micrographs of the ectoderm of the scyphistoma.....	126
Fig. 15 Electron micrographs of ecto- and endodermal cells in the scyphistoma.....	128
Fig. 16 Muscle and neuronal cells in the scyphistoma.....	130
Fig. 17 The ephyra of <i>Aurelia aurita</i>	149
Fig. 18 The muscle system of the ephyra.....	151
Fig. 19 Methylene blue staining of ephyral neurons.....	153
Fig. 20 Neurons labelled with anti-FMRFamide.....	156
Fig. 21 Anti-FMRFamide-labelled neurons in the ephyra.....	158
Fig. 22 MAb Aa label on striated muscle and in the tentacle.....	161
Fig. 23 MAb Aa label on the subumbrellar disc.....	163

Fig. 24 MAb Aa label on the manubrium and exumbrella.....	166
Fig. 25 MAb Aa label in the tentaculocyst.....	168
Fig. 26 MAb Aa label in the endoderm, and neurons stained with Rhodamine.....	170
Fig. 27 Rhodamine B stain and double-labelling in the ephyra.....	173
Fig. 28 Diagrammatic representation of distribution of neurons.....	175
Fig. 29 Electron micrographs of ectodermal cells of the ephyra.....	177
Fig. 30 Electron micrographs of ephyral muscle and nervous tissue.....	180
Fig. 31 Electron micrographs of endodermal cells in the ephyra.....	182
Fig. 32 Specificity of monoclonal antibody Aa.....	185
Fig. 33 Neurons stained with Rhodamine B after treatment.....	189
Fig. 34 Comparison of neurons stained with Rhodamine B after treatment.....	192
Fig. 35 Effects of treatments (FMRFamide, <i>Artemia</i> , glycine) on number of swimming beats of the ephyra.....	194

List of Tables

Table 1 Induction of metamorphosis in planulae of <i>Aurelia aurita</i>	92
Table 2 Effects of treatment (thyroxine, iodine, FMRFamide, and MgCl ₂) on metamorphosis of planulae.....	95
Table 3 Effects of treatments (thyroxine, iodine, FMRFamide, and MgCl ₂) on attachment of planulae.....	98
Table 4 Induction of metamorphosis with retinoic acid.....	101
Table 5 Effect of thyroxine on metamorphosis of operated animals.....	104
Table 6 Effects of treatments (MgCl ₂ and FM[D-R]Famide) on number of neurons stained with Rhodamine B.....	186
Table 7 Effects of treatments (FMRFamide and <i>Artemia</i>) on number of neurons stained with Rhodamine B.....	187
Table 8 Comparison of neurons stained with Rhodamine B.....	190

List of Abbreviations

Aa	monoclonal antibody generated using cells from the ephyra of <i>Aurelia aurita</i> as the immunogen; Aa recognizes a subset of neurons in the jellyfish stages of the life cycle.
BDH	British Drug House
BSA	bovine serum albumin
BCIP/NBT	bromochloroindolyl phosphate/nitro blue tetrazolium)
C	Celsius
cm	centimeter
d	day
DMSO	dimethyl sulfoxide
DNN	diffuse nerve net
ELISA	enzyme-linked immunosorbent assay
ETOH	ethanol
Fab	fragment antigen binding
Fig.	figure
FITC	fluorescein isothiocyanate
FM[D-R]Fa	phenylalanine-methionine-[D-arginine]-phenylalanine-amide
FMRFa	phenylalanine-methionine-arginine-phenylalanine-amide
FSW	0.22 μ m Millipore filtered seawater
g	force of gravity
GF	gastric filaments
GFNN	giant fibre nerve net
GFS	giant fibre system
GnRH	gonadotropin-releasing hormone
h	hour(s)

H ₂ O	water
H+L	heavy and light chain
I ₂	iodine
IP	intraperitoneally
KCl	potassium chloride
K ₂ HPO ₄	dipotassium hydrogen phosphate
kDa	kiloDalton
M	molar
mM	millimolar
mAb	monoclonal antibody
MgCl ₂	magnesium chloride
μg	microgram
μl	microliter
min	minute
ml	milliliter
mm	millimeter
NaCl	sodium chloride
Na ₂ HPO ₄	sodium phosphate dibasic
Na ₂ S ₂ O ₃	sodiumthiosulfate
NBD phalloidin	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phalloidin
nm	nanometer
NRA	non-relevant antibody
OsO ₄	osmium tetroxide
p	probability
Page	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	-log ₁₀ [H ⁺]

RPMI	tissue culture medium
s	second
SDS	sodium dodecylsulphate
SEM	scanning electron microscope
T₄	thyroxine
TEM	transmission electron microscope
Tris	(hydroxymethyl) methylamine

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For Karl Marx

and the rest of the clan
who thought higher education
wasn't for a woman.

To start at the beginning



Egg and sperm of *Aurelia aurita*

1. INTRODUCTION

The Life Cycle of *Aurelia aurita*

Aurelia aurita belongs to the Class Scyphozoa, Order Semaestomeae, Family Ulmaridae. The life cycle of *Aurelia aurita* consists of several developmental stages and includes an alternation of generations (Fig. 1). The fertilized egg develops into a free-swimming planula larva, which settles onto an appropriate substrate and metamorphoses into the sessile polyp or scyphistoma. The scyphistoma can reproduce asexually either by budding off other polyps or by strobilating, which results in the formation of ephyrae, the initial medusoid stage. In the strobila, ephyrae are produced by transverse fission and are stacked one on top of the other. Starting with the topmost one, the ephyrae are released and develop into the adult jellyfish. *Aurelia* is dioecious, and after fertilization has taken place internally, the female carries the developing planulae on her oral arms until their release. The planulae are uniformly ciliated and swim with their anterior (thickened) end pointing forwards. They are approximately 125 μm in length. The scyphistomae, which are less than 1 cm in length, are frequently attached to vertical or overhanging substrates, with their tentacles hanging downwards. The ephyrae, which are generally less than 1 cm in diameter, possess eight arms whose peripheral ends are bifurcated. The manubrium projects from the center of the subumbrellar disc. In the juvenile jellyfish, the oral arms, marginal tentacles, gonads and system of gastric canals begin to form, all of which are fully developed in the adult jellyfish. The latter can reach diameters of 35 cm.

Fig. 1 Life Cycle of *Aurelia aurita*

Free-swimming planulae released from the oral arms of the adult jellyfish settle and metamorphose into scyphistomae. By strobilation, the scyphistomae produce ephyrae, which develop into adult jellyfish.

P: Planula; S: Scyphistoma; St: Strobila; E: Ephyra;

J: Juvenile Jellyfish; A: Adult Jellyfish

Drawings are not to scale.

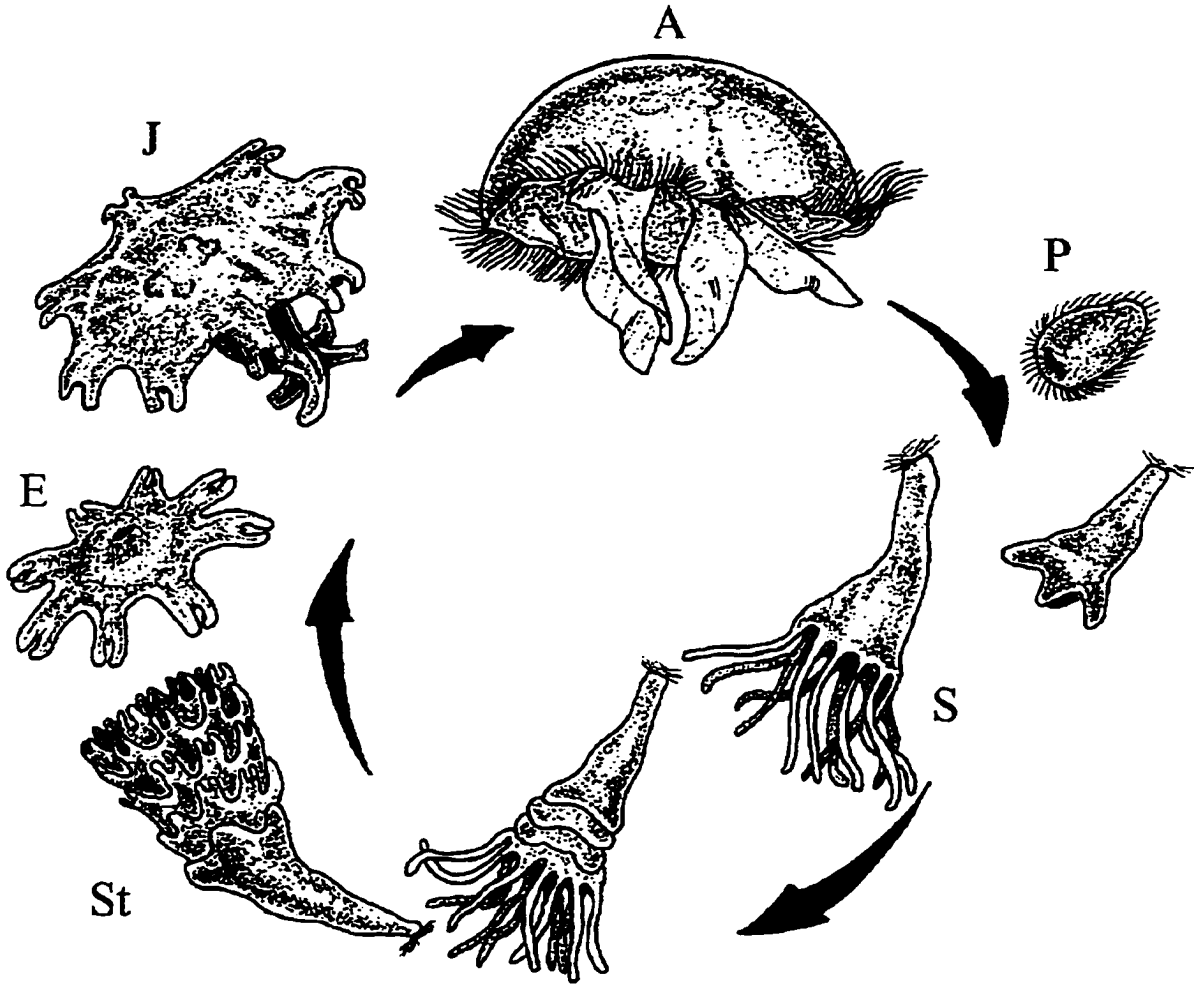


Fig. 1 The life cycle of *Aurelia aurita*

B. Historical Overview

The literature on coelenterates is extensive, and therefore a complete and detailed review, even one limited to neurobiological studies, is outside of the scope of this thesis. As the present work is concerned with the nervous system of the life cycle stages of *Aurelia aurita*, this overview concentrates, for the most part, on historical developments in the understanding of scyphozoan neurobiology, with emphasis on *Aurelia aurita*. It also includes reviews of the discovery of the scyphozoan life cycle, which chiefly occurred through studies on *Aurelia aurita*, and of classification systems. I have attempted to convey the different phases of scientific interest in coelenterates, and to place coelenterate research in the context of technological and intellectual advancements which contributed to its progress.

From Obscurity to Mainstream Biology

In terms of historical significance, the coelenterates are well represented in the scientific literature, even though, compared to such animal groups as the insects or the vertebrates, they were not focused on as early, or as much. Whereas the Scyphozoa have generally not received as much attention as the Hydrozoa (a trend which has persisted to the present), *Aurelia* figures prominently in the scyphozoan literature, which, however, concentrates on the adult jellyfish.

Around the turn of the nineteenth century, after their animal nature had been established, the coelenterates were mentioned mainly in the context of their position in the classification systems of the animal kingdom that were established at the time (Lamarck, 1801; Cuvier, 1795, 1828). In the first half of the nineteenth century, appreciation of their diversity increased (Péron, 1809), and interest in the coelenterates revolved around attempts at more detailed classification systems (Péron, 1809;

Eschscholtz, 1829), identification of their gross morphological components (von Baer, 1823), and understanding of their life cycle (Sars, 1841; Siebold, 1839). After Ehrenberg (1836) had reported "ganglionic swellings" in *Aurelia*, the acceptance of the presence of a nervous system in coelenterates, although gradual, led to a very prolific period in neurobiological research towards the end of the last century (1878 being particularly prominent, with four major contributions, *i.e.* by Romanes, Schäfer, Eimer, and the Hertwigs). Periods of intense research activity were often launched by the development of new methods and techniques, frequently pioneered in vertebrate research, *e.g.* for fixation and staining, and new or improved equipment, *e.g.* microtomes and microscopes. In addition to technical advancements, intellectual developments, such as the development of the concept of cellularity in the mid-nineteenth century and the acceptance of Darwin's theories, are also reflected in the development of coelenterate (neurobiological) research and fueled general interest in coelenterates. Haeckel, for example, in the latter part of the nineteenth century, worked on a classification system for the coelenterates, studied coelenterate life cycles, and, as an avid supporter of Darwin, emphasized the importance of coelenterates in studies concerned with phylogeny and the ancestral metazoa.

In terms of evolutionary significance, the coelenterates figure prominently in the discussion on the origin of metazoan phylogeny, since, at the tissue level, their organization is thought to approximate the ancestral condition of the first metazoa. Their nature and systematic position, however, were long debated. Aristotle called them *Acalephae* (Gr. *akalephe*, nettle) or *Cnidae* (Gr. *cnidos*, thread) because of their ability to sting, and placed them between the plants and animals, amongst the *Zoophyta* (Gr. *zoon*, animal; *phyton*, plant), a term that was retained until about a century ago (Hyman, 1940). Abraham Trembley (1710-1784), (Lenhoff, 1979) who worked on freshwater polyps, was the first to clearly realize their animal nature. He observed their movements, food uptake and propagation, and carried out extensive experiments

regarding their ability to regenerate. Subsequent researchers, such as Linnæus (1758), Lamarck (1801), and Cuvier (1828), placed the coelenterates among animals, in their groups Radiata or Zoophyta. In the latter, however, the coelenterates were, on the one hand, included with a variety of other invertebrates while, on the other hand, they were split into the medusae and the polyps. Studies on the life cycles of coelenterates, such as the ones by Sars (1829) on *Aurelia*, demonstrated the relationship between polyps and medusae and the fact that they did not belong to different groups. Frey and Leuckart (1847), recognizing the common body plan of all polyps and medusae, introduced the term 'Coelenterata' (Gr. koilos, cavity; enteron, intestine). The sponges, however, were still included in this group, whereas the coelenterates proper were referred to as 'Acalepha'. Huxley (1849), who recognized that the medusae are diploblastic, consisting "essentially of two membranes", and that they shared this characteristic with the polyps, further stressed that the medusae and the polyps "are members of one great group, organized upon one simple and uniform plan". [He also stated, however, that he had "not observed any indubitable trace of a nervous system in the medusae".] Hatschek (1888) split the coelenterates and distinguished three phyla, the Spongiaria, Cnidaria, and Ctenophores. Zoologists who prefer the combination of coelenterates and ctenophores refer to the phylum as 'Coelenterata' and divide it into the Cnidaria and the Acnidaria, *i.e.* the ctenophores, although nowadays, 'Coelenterata' is frequently used synonymously with 'Cnidaria', and the ctenophores are regarded as a separate phylum. The Cnidaria are divided into the Hydrozoa, Anthozoa, and Scyphozoa, the latter receiving their name from Haeckel (1891) (Hyman, 1940). Relatively recently, the cubomedusae have been separated from the Scyphozoa and placed in their own class, the Cubozoa (Werner, 1975), although it remains to be seen if this gains acceptance.

It was not until the 1820s that the first intense period began with studies focusing on coelenterates, and the emergence of coelenterate biologists, (which is not to

imply that they did not work on other subjects as well). More often than not, 'coelenterate biologists' of the time had received medical training (and had a 'vertebrate-frame-of-mind', which explains their approach, *i.e.* the application of vertebrate terminology and identification of vertebrate structures, to the investigation of the previously virtually unexplored group of 'lower' marine invertebrates) and their body of work included a variety of studies, ranging from protozoa to mammals and plants. von Baer (1823) and Eschscholtz (1829) were among the first scholars of coelenterate biology, providing a detailed description of *Aurelia* and a classification system of the medusae, respectively. There *was* an earlier effort to establish a system of the medusae (Péron, 1809), but it is more a compilation of descriptions and locations of the medusae Péron encountered. He was, however, the one to give *Aurelia* its name, and his choice prevailed over others such as *Medusa*. While several of the names suggested by Péron (1809) are still used, albeit sometimes in a different context (he introduced the term 'ephyra', but as a genus name, for example), it is not easy to find a given species in his system (*Aurelia*, for example, is distributed over several genera). In comparison, whereas the use of some of Eschscholtz' (1829) terms, such as his Discophorae (which included the scyphozoan species), has been discontinued, his system is more user-friendly by today's standards, as medusae that he grouped together have often remained so until today, *e.g.* the ctenophores. Péron's contribution to the emerging field of coelenterate biology is not the system he provided, but stems more from the fact that, given his advantage of encountering numerous collection sites and his descriptions of fresh specimens, he could introduce scientists without the opportunity of extensive travel to the diversity and distribution of the medusae. While Péron described the gross morphology of the animals and their place of origin, Eschscholtz (1829), as had von Baer (1823) before him, dealt more with the biology of the medusae, providing more morphological details and suggestions for their identity and function. They were not necessarily always right, as when Eschscholtz attributed

the function of a liver to the tentaculocysts, however both were not just concerned with a description of the adults' body parts, but also debated their mode of reproduction and the form of their young. The debate was taken one step further by Sars, whose study, begun in 1829 and continued over the next decade, led to the elucidation of the scyphozoan life cycle through careful observations, rearing experiments, and familiarity with the literature. The studies by Sars and later by von Siebold (1839) represent the culmination of this first period of recognition of the coelenterates: from the recognition of their animal status, diversity and their interrelationships, to the beginning of the identification of their morphological structures and the discovery of their life cycles. Later studies, benefiting from technological and intellectual advancements, provided more detailed descriptions and placed the coelenterates in a phylogenetic context. Even after all of the stages were known, however, more studies have dealt with the adult jellyfish than with any other stage of the life cycle not only of *Aurelia aurita*, but generally of scyphozoans.

Classification Systems

After Trembley's pioneering work in the mid-eighteenth century, widespread interest in the coelenterates from the scientific community was slow to develop. With their animal nature established, the coelenterates could not be ignored in classifications of the animal kingdom, and were included in both Lamarck's (1801) and Cuvier's (1795, 1828) systems. The attempts to categorize the animal kingdom around the turn of the 19th century represent the beginning of detailed comparative anatomical studies that subjected the coelenterates, for the first time, to the same consistent criteria as the other animal groups. This was an important first step, even though *Aurelia*, for example, was not only given different names (*e.g. Medusa* or *Cyanea*), but was also assigned to different families and even classes. Both Cuvier (1828) and Lamarck

(1801) expanded on the view commonly held by many naturalists of the time, including Linnæus (1758), to divide the invertebrates into only two classes, namely the insects and the worms (the coelenterates were grouped in an order of the latter class, the Zoophyta, a term which Lamarck objected to "as their nature is completely animal, and in no respect vegetable"). They both based their respective systems on comparative anatomical studies not only of contemporary species, but also of fossils, and their treatment of the coelenterates is similar: they both placed them last and separated the jellyfish from the polyps. Neither would probably appreciate a statement pointing out these similarities, given Cuvier's and Lamarck's adherence to opposing philosophies: Cuvier believed in catastrophism and immutable species, whereas Lamarck believed in a gradation of species and change over time.

Cuvier (1828), drawing from his own studies and those of many naturalists, among them Lamarck, attempted an organization of the animal kingdom, the first result of which he published in 1795, and which he continually updated, most notably in 1816 and again in 1828. He acknowledged, however, that his treatment of the invertebrates was a mere modification of the divisions in his 1795 memoirs. Cuvier (1828) organized the animal kingdom into four divisions, based on the correspondence of general forms, which "results from the arrangement of the organs of motion, the distribution of nervous masses, and the energy of the circulating system". The divisions thus formed consisted of the vertebrate, molluscan, articulate and radiate animals, and these were subdivided into classes, orders, genera, and species. As to the latter, Cuvier (1798) emphasized that variable properties, most importantly size and colour, should be recognized as variations, which were frequent in the same species. Cuvier (1828) recognized four classes of radiate animals, which "have no distinct nervous systems, nor organs of sense". He placed *Aurelia* in the third class of the radiata, (the acalepha), in the acalepha simplicia, the first of two orders. Although Cuvier also named a genus *Medusa*, whose subgenera (*e.g. Aequorea* and *Pelagia*) lack

lateral cavities, he placed *Aurelia* in the genus *Cyanea*, whose species have a central mouth and four lateral cavities, and called it *Cyanea aurita*. Cuvier must have observed some developmental stages, since he stated that, "with age, *Cyanea aurita* acquires four very long arms" [the oral arms of the adult jellyfish]* and that, in the *Medusa* subgenera, "the tentacula, whether on the margin of the umbrella, or round the mouth of the animal, vary not only in different species, but in different ages of the same species". He further observed that in the majority of *Medusa* subgenera with a simple mouth, "there are, in the substance of the umbrella, four organs enclosed in furrowed membranes, which, at certain seasons of the year, are tinged with a dark-coloured substance, understood to be the germs of the young". Cuvier was also the first to note that the body of the polyp is "frequently without any viscera but its cavity and frequently with a visible stomach and intestinal tubes, which are hollowed out of the substance of the body, as in the medusae". Although he had discovered that the polyps shared with the medusae the fact that the stomach was not a separate organ within the body cavity, but was itself the body cavity, which served for digestion as well as circulation, he did not go as far as placing the medusae together with the polyps in a group of their own and with the exclusion of other animals. It was not until 1847 that Frey and Leuckart, who recognized the homologous arrangement of the body cavity (coelom) and gut cavity (enteron) in polyps and medusae, proposed to name such a united group 'Coelenterata'.

In his 'Système des animaux sans vertèbres', Lamarck (1801), who was the first to separate the vertebrates from the invertebrates, based the division of the invertebrates on their organization, in particular the organization of the organs of respiration, circulation, and "sentience", and thus arrived at seven classes: the mollusks, crustaceans, arachnids, insects, worms, radiate animals (which contained the jellyfish), and polyps. His 'radiaires' consisted of two orders: the echinoderms and the

*Notes in brackets [] represent comments, clarifications, and translations by this author.

'radiaires molasses'. Some of the characteristics of the latter were: a completely or partially gelatinous body, transparent skin, and no appearance of nerves. 'Méduse' formed the first of nine genera, and he described his '*Medusa aurita*' as having a free body with a convex upper and a concave under side and an inferior, central and single mouth.

Both Lamarck (1801) and Cuvier (1828) distinguished three orders of polyps, but whereas Lamarck placed them in a class of their own (albeit the last of his seven classes of invertebrates, following the 'radiaires'), Cuvier positioned them in the fourth class of his fourth division, the radiate animals, behind the class acalepha. In either case, neither of them appears to have observed a scyphistoma. Attempts to locate scyphistomae among the described animals are somewhat difficult since the authors did not consistently provide illustrations or measurements, and the written descriptions are sometimes vague. However, Cuvier's definition of the class includes: propagation by putting out buds, but also by eggs, which excludes scyphistomae. Lamarck treated the polyps in more detail than Cuvier, and one could speculate that, if he had described them, scyphistomae would most likely be found in the first of the three orders. Lamarck divided the latter into 'polypes à rayons nus', which includes *Actinia*, and 'polypes à rayons coralligènes', and it is the description of the former that could best fit a scyphistoma: naked or uncovered polyps that are fixed at the base, have great regenerative powers and that multiply by budding.

I have made mention of the above systems as they represent the thinking of the time and the starting point for subsequent and more detailed systems. It was with attempts at classification systems of the medusae that the coelenterates (or at least some of their members) came into their own. Having been included amongst the animals, the different species of coelenterates now needed to be described, catalogued, and classified. Whereas both Lamarck and Cuvier rarely described more than one species per genus, Péron (1809a) prided himself in having compiled a list of medusa species

more than ten times longer than any before (his list, however, contained a number of questionable and probably superfluous genera). Péron, who was the resident scientist on one of the ships of a two-ship expedition that lasted several years, and who was accompanied by an artist to assist in the illustrations, was, to my knowledge, the first to expand on the general system of the animals and to focus his publication solely on the medusae. However, one cannot help but get the impression that his priority was not only to obtain as many different specimens from as many different locations as possible (which in itself is commendable), but also to create as many new genera and species as possible, which he tried to fit into a rigid system.

Lamarck (1801) and Cuvier (1795, 1828) had based their respective classification on the organization of the animals' organs, and even though they applied vertebrate terminology, such as Lamarck's 'organ of sentience', to invertebrates, Cuvier had already pointed out that variations, particularly those in size and colour, should be recognized as such, and occurred in the same species. Péron, on the other hand, classified the medusae based on the presence or absence of body parts, and seems to have been preoccupied with the maintenance of symmetry in his, mainly dichotomous, system. He must have formulated his system prior to encountering representatives for his divisions, as some of his groups are void of examples. His list of species and genera, on the other hand, is longer than any before, chiefly due to his introduction of new species based on differences in size, colouration, or location. [I do not know the extent of his training, but surely, as a Frenchman, he must have been familiar with Lamarck's and Cuvier's works?] Of the medusae, he noted that their mode of reproduction was not known with certainty; details of their muscle system were unknown; and their system of nutrition escaped him.

Péron's (1809b) two main divisions of medusae consisted of those with and without a distinct stomach. He separated each group into those with or without peduncles, each of which he further subdivided into those with or without tentacles. In

addition, the second division, *i.e.* the medusae with a stomach, contained two sections, the monostomes and the polystomes, and were further distinguished by whether they did or did not possess arms. Consequently, some of his categories were devoid of examples. On the other hand, it appears that a slight difference in colouration or a different place of occurrence presented enough of a criterion for Péron (1809b) to establish a new species. For example, he introduced ten species of '*Aurellia*', which he placed amongst the polystomes with composite stomachs, no peduncle, but arms and tentacles, and which he described as having four mouths, four stomachs, four ovaries, four arms, an air cavity ? [sic] in the centre of the umbrella, and eight little processes on their circumference. '*Aurellia purpurea*', which Péron identified as a synonym of '*Medusa aurita*', has an orbicular umbrella, a "pretty" purple colour, and exists off the coast of Biscay, while '*Aurellia rosea*' is predominantly rose-coloured, 10 cm in diameter and is found in the Baltic sea. Other species are one cm in diameter and from the Mediterranean (*Aurellia rufescens*) or from the port of Naples (*Aurellia amaranthea*). One wonders whether Péron (1809) observed the same animal at least in some of the different locations he passed through during his travels.

In addition to introducing new species, Péron probably also described *Aurelia* in his genus '*Evagora*', which he characterized as having four ovaries that form the shape of a cross or a ring. '*Evagora tetrachira*' has four white ovaries, four strong and lancet-shaped arms, is five to six cm in diameter and lives in the Mediterranean sea. Péron also introduced the genus '*Ephyra*', whose stomach has four simple openings that are opposed two by two. However, he could not have referred to the young of *Aurelia*, since his two '*Ephyra*' species are 24 cm and 25-30 cm in diameter, respectively.

Péron's (1809b) classification was criticized by von Baer (1823), who referred to *Aurelia aurita* both as '*Medusa aurita*' and as '*Aurellia aurita*', and who stated that: "Péron, drawing from murky sources, not only divided [*Aurelia*] into many species, but several genera", and that Péron's genus '*Evagora*' was hardly different from

[*Aurelia*]. von Baer described *Aurelia* in detail and emphasized that it only possessed one stomach and one mouth. He suggested calling the opening created by the four arms *Schlund* [pharynx] rather than mouth, since it formed a transition between the mouth cavity [the space surrounded by the trailing edges of the arms] and the stomach. He observed the branched and unbranched canals originating from the stomach and saw that they, too, just as the outer surface of the animal, were lined by a "skin". He further noted eight small "grains" [tentaculocysts] of unknown function which divided the margin of the animal into eight lappets. von Baer (1823) also identified the ovaries and eggs of *Aurelia*, and debated the identity of the "small moving bodies" [probably planulae] on the oral arms. He dismissed his [own] first suggestion, that they were eggs, since they were moving, and his second, that they were parasites, since there were too many of them. He thought it most probable that they represented not yet developed medusae, and since they differed in form considerably from the adult medusa, he called them larvae. von Baer (1823) left the question regarding their fate to be decided by future research, but suggested that they could either gradually transform into medusae, or undergo a less gradual metamorphosis.

Eschscholtz's (1829) 'System der Acalephen' provided "a comprehensive description of all medusa-like radiate animals", a third of which he had examined himself (73 out of 200). He based his definition of the class Acalepha "on their way of life, since their need to swim freely is directly related to their feeding habits", and therefore rejected the idea to include polyp-like animals in the class.

There are three orders in Eschscholtz's system: the Ctenophorae, Discophorae and Siphonophorae, and two divisions in the Discophorae. In the first division, the genus *Medusa* of the family Medusidae has nine species, among them *Medusa aurita*. He could not determine how the contraction of the disc was produced, since he did not see any muscles or muscle fibres, and thought the animals lacked a nervous system. He distinguished between the tentacles of the disc and those of the oral arms, and

insisted that they had to be covered by invisible "warts" [probably nematocysts], since they constantly adhered to foreign objects. He ascribed the eight small "grains" [tentaculocysts] in the margin of the disc the function of a liver, since they appeared to be gland-like. Eschscholtz (1829), too, observed the opening of the mouth between the oral arms, the numerous canals emanating from the stomach, and the egg-filled bulges associated with the under side of the digestive apparatus. He also saw the "seeds" [probably planulae] on the oral arms and assumed that the medusae were annual animals, since he found their brood in the spring and large animals in the fall.

One of the new species of medusa Eschscholtz introduced was in the family Ephyra: *Ephyra octolobata*, which he characterized as: *marginē disci lobis octo magnis apice bifidis*. He found a single specimen in the Atlantic Ocean, near the equator. The figure he provided for the animal confirms that he had seen an ephyra larva, although the branching of the endodermal canals is drawn incorrectly. He speculated that the animal was probably a young one, which later in life could develop oral arms and tentacles. The other two species in the family correspond to species already described by Péron (1809) [*Ephyra tuberculata* (25-30 cm), and *Euryale antarctica* (74-80 cm), which Eschscholtz renamed *Ephyra antarctica*], and could not have been ephyra because of their size.

Discovery of the Life Cycle

So far, then, as far as the different developmental stages of *Aurelia* are concerned, Cuvier (1828) had recognized the eggs, von Baer (1823) the planula larvae, and Eschscholtz (1829) had seen the ephyra. Also in 1829, Michael Sars (Winsor, 1976) described a new species of polyp, *Scyphistoma filicorne*, which was only a few millimeters tall and had 20-30 tentacles around its mouth, and a new species of medusoid jellyfish, *Strobila octoradiata*, which had a deeply lobed disc and which he

saw originate from a stack of such discs. Sars recognized the similarity between *Strobila* and Eschscholtz's (1829) *Ephyra*, and conjectured that *Strobila* was a young *Ephyra*. In 1835, Sars (Winsor, 1976) published observations he had made in 1830 of animals that he had seen swimming amongst *Medusa aurita*, and which he thought to be older forms of *Strobila*, since they were larger and had less pronounced indentations in their discs.

Wiegmann (1836) recognized Sars' work in his "report on the accomplishments in the field of zoology". He expressed his utmost surprise at Sars' discovery that *Scyphistoma* was an earlier stage of *Strobila*. Reporting Sars' findings, Wiegmann (1836) stated that "the medusa in its first stage has the appearance of a polyp", which is sessile, gelatinous, cylindrical and broadened at its oral end. The latter possesses 20-30 tentacles that are the length of the body. The tentacles move in all directions and shorten when touched without retracting into the body. The mouth can open almost to the width of the body, which is hollow and without intestines. In the second stage, deep folds appear at equal distance from one another, increasing in numbers as the animal develops. In the third stage, each fold develops short lappets with bifurcated ends. The lappets of one animal are situated exactly below the ones of its neighbour, and are all directed upwards. The convex side of the lowest animal is elongated into a stalk with which the whole "family" is attached. Separation occurs in the fourth stage, when the ephyra-like animals are released starting from the top downwards. According to Wiegmann (1836), Sars observed the separation of 14 completely developed animals, whose convex sides connected to the concave oral sides of the lower neighbours, without, however, forming an "organic" connection. The organization of the released animals is that of the genus *Ephyra*, and Wiegmann suggested giving up the genus *Strobila*, since it only described a developmental stage, and the animals resulting from it could hardly be generically different from *Ephyra*.

In 1837, Sars collected a series of specimens representing transitions from *Strobila* up to a small *Medusa* and believed that these animals were all part of the life cycle of *Medusa aurita*. He had hoped to demonstrate his observations with a series of illustrations at a conference in Prague, but complained that he was unable to because the other speakers had taken up too much time. In 1839, Sars (Sars, 1841) was successful in rearing some planulae from *Cyanea capillata*, which he had obtained from adult females, and observed them metamorphosing into young polyps that were indistinguishable from his *Scyphistoma*. Sars was thus able to reconstruct the entire life cycle of *Medusa aurita*, but it was von Siebold (1839) (Sars, 1841) who published it first. Sars (1841) was left to confirm Siebold's "beautiful" work and emphasized that he had made his observations independently from von Siebold.

Sars (1841) conceded that he only had the use of an inferior microscope, but nevertheless he provided extremely detailed and precise descriptions and illustrations. He also supplied measurements, dates and locations of his collections. Sars began his paper by proving that *Strobila* is a young stage of *Medusa aurita*, which he felt he still owed to the scientific community, since he had been unable to do so at the 1837 conference in Prague. He had observed a number of small medusae, some of which resembled the newly released *Strobila*. They possessed a flat disc that became hemispherical when contracted, eight bifurcated arms each with a tentaculocyst at the base of the lappets, and a long, square or tube-like mouth protruding from the subumbrella, and no tentacles. He even observed the gastric filaments, and complained that the artist who had provided the drawings for an earlier publication had misrepresented the gastric canals. In his figure and description of the Norwegian animals, one somewhat wider canal extends from the stomach into each of the arms, branching at the base and again just below the tentaculocyst, and another, shorter and narrower canal reaches between any two arms. [In the just released ephyrae of the Pacific Ocean, the shorter canals are wider and the longer canals do not branch at the

base.] He documented the gradual transition from the ephyra to the adult jellyfish with a number of drawings, demonstrating that the space between the arms diminishes, tentacle buds appear at the margin of the disc, and the gastric canals continue to branch out. The edge of the mouth acquires tentacles, too, and starts to divide into four arms. As development proceeds, the separation of the arms continues until, in the adult jellyfish, they are only connected at their base. Sars also observed "papillae" [probably nematocysts] on the exumbrellar surface and could see the movement of particles in the gastric canals below the tentaculocysts. Apparently he only saw the "calcium carbonate" crystals [they are actually calcium sulfate] in tentaculocysts of adult animals.

Whereas Sars did not have the opportunity to confirm but agreed with von Siebold's (1839) findings that the animals are dioecious, both observed the free-swimming, uniformly ciliated, oval planulae, which Sars likened to the larvae of other polyps. He saw the planulae swimming with their widened end forward and attach themselves with the same end onto the glass or water surface. The free end then broadened and developed a mouth and tentacle buds. Some of the animals almost doubled in size within three days. Tentacles continued to develop, and Sars could see the same kind of "papillae" on their surface as in the jellyfish. The interior of the body was divided by four ridges or septa, the continuations of which appeared as four round "holes" around the mouth. Sars frequently found small plankton inside the animals, and observed that both the body and tentacles contracted upon stimulation. Sars expressed his amazement at the polyps' ability to reproduce in two ways. He followed the development of buds originating from different parts of the body, and that of stolons, growing usually from the bases of the polyps along the substrate, until they, too, produced bud-like extensions. Sars found that these types of reproduction occurred even before the onset of strobilation.

According to Sars (1841), Dalyell's (1836/7) observations of buds, as well as of the production of free-swimming medusae by horizontal divisions, confirmed Sars' own observations. He claimed that Dalyell's *Hydra tuba* was none other than *Strobila*, but disagreed with Dalyell as to the fate of the polyp after the release of the ephyrae. Sars never saw the reappearance of tentacles and speculated that both they as well as the rest of the polyp body disappeared, whereas Dalyell [rightly] maintained that the polyps formed new tentacles and continued on. Having demonstrated that the animals really all belonged to the life-cycle of *Medusa aurita*, Sars proposed to remove the genera *Strobila* and *Ephyra*. In addition to *Medusa*, Sars (1841) was also able to examine *Cyanea capillata* and pointed out that their development is basically the same. I find it surprising that Sars did not mention any muscles in his descriptions, since he clearly saw and drew both radial and circular muscles in the figure of a young *Cyanea* (Sars, 1841, table IV, figure 64).

In his conclusion, Sars stated that "it is not the larva or the individual developing from the egg that develops into the perfect medusa, but the offspring of that larva; it is not the individual but the generation that metamorphoses." Chamisso (1819) had termed this process of change in form, where a whole generation differs from the previous one, 'alternation of generations' (Sars, 1841). Steenstrup (1842) (Winsor, 1976) described the life cycle of *Medusa aurita* in detail and as an example of reproduction by an alternation of generations. In his view, the first generation should not be called larvae, like the first stage in an insect's metamorphosis, because it does not grow further. Instead, he used the term "Amme" ([wet]-nurse), because it produces a new brood of individuals, of a different form, which represents the second generation. According to Steenstrup, the scyphistomae are therefore nurses to the second, free-swimming medusoid generation.

Since its initial discovery, several studies on various species of Scyphozoa have provided more details to the life cycle. Agassiz (1862), who coined the term

'Semaestomeae', described the formation and growth of *Aurelia* both morphologically and microscopically (he was able to achieve magnifications of 500 times). Most of the observations were his own except for the ones on "the eye", which were by Clark. The latter mistook the contents of the statocyst as a "true crystalline lens" which "suberves the purposes of actual vision", (Agassiz, given that he published it under his name, must have agreed) but made no mention of nerve cells. Agassiz emphatically defended his position that the coelenterates and the echinoderms are "so completely built upon one and the same plan" (he homologized the "system of radiating tubes", for example) that it was out of the question to regard them as two distinct primary divisions (according to Haeckel (1882), Agassiz was Darwin's greatest opponent.)

Haeckel himself (1881) reexamined the life cycle of some Scyphozoa, including *Aurelia*, *Pelagia*, and *Chrysaora*. He noted that, under certain conditions, both *Pelagia* and *Aurelia* developed directly, without passing through the scyphistoma and strobila stages. Although he described the development from the scyphistoma through the strobila to the ephyra, he made no mention of the adult jellyfish. Claus (1883) disputed Haeckel's (1881) statement regarding the direct development of *Aurelia*, and instead emphasized that, under normal circumstances, *Aurelia*'s development was remarkably constant. [Claus was right. It is a different story for *Pelagia*, however. *Pelagia colorata*, as *Aurelia*, goes through an alternation of generations, whereas *P. noctiluca* develops directly.]

Progress in Neurobiology

Although the entire life cycle was known by the mid-nineteenth century, the trend to focus on the adult jellyfish continued and is particularly evident in research on the nervous system. Why this should be so is not entirely apparent. The adult jellyfish is, to this day, referred to as the 'dominant' stage of the life cycle (Barnes, 1987), but

what does this dominance consist of? It is, in size, more conspicuous than the other life cycle stages, but it is also seasonal, which the scyphistoma, at least, is not, and is outnumbered by the scyphistomae, planulae and ephyrae, although the latter two occur for short time periods only. So whereas the scyphistomae can be found year-round, they are less conspicuous, and perhaps their small size made them less desirable study objects.

Whereas some studies of that time period focused on neurobiological research, results of the latter were supplemented by those from whole animal or life cycle studies (von Lendenfeld, 1882; Agassiz, 1862). The latter reported nervous structures in the jellyfish (at least if one, as for Agassiz, considers "the eye" a nervous structure) but not in the scyphistoma, and perhaps neurobiologists deemed its behaviour not interesting enough to warrant further investigation. Compared to either the scyphistoma or the adult jellyfish, the ephyra, whose behaviour is very similar to that of the latter, is a lot more difficult to find, however, the task is not impossible. Why it took until 1956 for the first concentrated effort (Horridge, 1956a) to examine the nervous system of the ephyra remains a puzzle. Whatever the reasons may have been, the neurobiologists concentrated on the adult jellyfish, and therefore so does the following account of their progress.

The commonly held view at the end of the eighteenth and the beginning of the nineteenth century was that the medusae did not possess a nervous system, although in 1775, Forskål (Hertwig and Hertwig, 1878) had interpreted the red stripes of *Pelagia* as nerves [which they are not]. Who exactly was the first to - correctly - identify a nervous system in medusae is a matter of debate. Whereas Forskål not only described the wrong area (stripes on the exumbrella), but also 'wrong' structures (nerves), later accounts may have identified right areas, but whether they identified the right structures is questionable. Ehrenberg (1836) was the first to positively attribute nervous structures to the margin of the medusae. One could speculate that Ehrenberg, who had

studied medicine and also participated in several expeditions to the East, was guided to his discovery by his belief that all animals, including unicellular organisms, descended from one ancestral type and thus should all possess the same kind of organ systems. He expected them to be as "vollkommene" [complete] organisms as possible, and as it turned out, he was right with regard to a nervous system in the medusae [he had, however, also assumed that the nuclei of infusoria represented sexual organs (Nordenskiöld, 1946)]. Whatever his motivation may have been, he described ganglionic swellings, which included the tentaculocysts, in *Aurelia aurita*, and thereby initiated a new period of research, where, gradually, the existence of a nervous system in medusae was more generally accepted.

Ehrenberg (1836) had definitely described the right area, however, as Eimer (1878) pointed out, Ehrenberg's observations may not have been of ganglionic swellings after all, but rather of ectodermal folds in the area of the tentaculocyst. Similarly, Agassiz' (1850) conclusions regarding the presence of a nervous system in Hydrozoa were, according to Romanes (1876) "certainly unwarranted by the facts" and "decidedly premature". Agassiz had described the medusan nervous system as consisting of a simple cord forming a ring around the lower margin of the animal. The cord was cellular throughout with no appearance of fibres. Again, the area was right, but Romanes maintained that Agassiz' structures were the "optical expression of a thickness of ectoderm in the region of the nutritive canals". Romanes himself was a cautious man. In the first (1876) of a series of papers, he stated that "the only legitimate attitude of mind to adopt towards the much-vexed question as to the presence of nerves in medusae, is that which is thus tersely formulated by Huxley (1849): 'no nervous system has yet been discovered in any of these animals.' " Nevertheless, Romanes did state that "it is to the medusae we must look for the first decided integrations of tissue having, to say the least, something resembling a nervous function to subserve". Two years later, after extensive studies on *Aurelia* and some other

medusae, his results would have the most pronounced impact on the understanding of the medusan nervous system. With a series of ingenious cuts and the application of various stimuli (he was the first to apply electrical stimulation to *Aurelia*), he had "raised the [tentaculocysts] to the dignity of locomotor centres", deduced that "there exists a more or less intimate plexus of lines of discharge" in the subumbrella (although he assumed the presence of anastomoses), observed two waves of contraction, [one of which, that of the subumbrellar muscles, traveled twice as fast as that of the marginal tentacles], and inspired a histological study carried out by Schäfer (1878), who was the first to successfully stain neurons of *Aurelia* with gold chloride. All these accomplishments by Romanes, which could let one -almost- overlook the fact that he did not follow up his physiological experiments with some histological observations of his own, particularly since he was very aware of their importance, are certainly impressive and indicative of his 'coelenterate frame of mind'.

It is not surprising, given the circumstances of the time, that some researchers, such as Huxley (1849), expressed their skepticism for the existence of a medusan nervous system. In this early period of coelenterate neurobiology, researchers attempting to find elements of a nervous nature in medusae were not only limited by the equipment and methods available, but also by the scientific thinking of the time. The cell theory was still in its infancy, and the cell's contents, formation and boundaries (did it have a membrane?, were structures such as muscle fibrils foreign to the cell?) were still being debated. The fact that nerve cell body and fibre were connected was not established until the late 1840's (Stricker, 1871). What was mostly associated with the physical manifestation of a nervous system were nerves, *i.e.* bundles of fibres, which are characteristic for vertebrates and higher invertebrates but are rare to absent in coelenterates. If prominent nerves were proof for the presence of a nervous system, any search for them in medusae would more likely than not have led to the conclusion that medusae did not possess a nervous system.

Recognition of nervous structures not only depended on the expectations of the researcher, but also on the methods used. We know that Ehrenberg, who had discovered the myelinated nerve fibre in 1833 (Ramón y Cajal, 1897, 1984), had used dissection and a microscope. It must have been difficult, given the preparation techniques and microscopes of the time, to try and locate nervous structures in adult *Aurelia*. Dissection of live animals in order to obtain a piece of tissue of a small enough size appropriate for the microscope can be frustrating, given the amount of mesogloea involved, and, for the same reason, obtaining of sections must have been virtually impossible, since customarily, prior to the introduction of the microtome by His in 1870 (Nordskiöld, 1946), tissue was sectioned by clamping it between pieces of cork or leather and sectioning it by hand. Perhaps that is why Ehrenberg (1836) reported only "ganglionic swellings" [if that is indeed what he saw], as there is an accumulation of nerve cells in the tentaculocyst, and the surrounding area is flatter and is easily excised. Even with modern microscopes, unstained neurons are not always readily identifiable, and, while the problems of spherical and chromatic aberration in microscopes were understood in the 1830's (McCormick, 1987), high resolution microscopy was only achieved in the late 1860's (Turner, 1980). By the 1870s (Stricker, 1871), magnifications of 1000-1500 times were possible, and humid chambers and heatable stages were available. Also, frozen sections and embedding in wax was possible. The most dramatic development, however, occurred in the introduction and improvement of histological techniques. The use of vinegar and alcohol as fixatives had been known in the 18th century, when acetic acid was used to preserve hydras (Humason, 1972), and a large number of chemicals for fixation, hardening, and staining purposes would be introduced in the 19th century. In the 1830s, Heinrich Müller introduced potassium dichromate, and Max Schultze, in the 1860s, osmium tetroxide (Nordskiöld, 1946). Osmium tetroxide (alone or together with acetic acid) and potassium dichromate were used for fixation, hardening, and

maceration of tissue. In 1858, Gerlach (Hertwig, 1929) introduced carmine as a general stain, and in 1863, Waldeyer introduced hematoxylin and Benecke, aniline dyes (Nordskiöld, 1946). Whereas stains such as carmine were successfully used by coelenterate biologists (Hertwig and Hertwig, 1878, Krasinska, 1914), the discovery of dyes with a high affinity for neurons revolutionized neurobiological research. Metallic impregnation was introduced first. Gerlach used gold chloride in 1858 on the spinal cord (Lee and Mayer, 1907), but it was Golgi's method of using silver nitrate on the brain, first published in 1873, that produced clear and decisive images of cells, and would inspire others, most notably Ramón y Cajal, to attempt metallic impregnation (Ramón y Cajal, 1984). Another stain which is used to this day, the vital dye methylene blue, was introduced by Ehrlich (1886), and, in its reduced form, by Unna (1916). These specific dyes had a great impact on neurobiological research in general, and also on coelenterate research. However, the impact on the latter stemmed perhaps, at least initially, more from the fact that results from using the dyes were used as arguments in the general discussion about continuity-versus-contiguity of neurons, which was also debated for coelenterates. Many prominent coelenterate biologists, such as the Hertwigs (1878), Krasinska (1895), and Bethe (1903), continued to use more general staining techniques. Schäfer (1878) was the first to stain *Aurelia's* neurons with gold chloride, and, in describing the bipolar neurons of the subumbrellar nerve net, stated that their processes ended freely. This evidence, however, did not convince Bethe (1903), who was of the opinion that Golgi's method and methylene blue were unsuitable for settling the continuity-versus-contiguity argument, since neither stained all the nerve cells that were present at the same time [which is exactly what had excited Ramón y Cajal]. Thus, he reasoned, existing connections were simply overlooked. He preferred a general stain, and used molybdenum and toluidine blue. Bethe believed that the coelenterate nerve plexus consisted of a net of multipolar ganglion cells whose processes anastomosed. This view was still upheld by Fortuyn

(1920), who therefore concluded that Schäfer (1878) could not possibly have seen the entire length of the processes. Bozler (1927), who used reduced methylene blue, attempted to solve the question of continuous versus individual cells and found that none of the fibres of *Rhizostoma*, whether they belonged to bipolar, multipolar, or endodermal neurons, ever fused, but that they always remained separate.

With the acceptance of evolutionary concepts, new questions arose and the debate on the nature of the structural elements of the coelenterate nervous system was expanded to include phylogenetic considerations. Kleinenberg (1872), who used various acids (acetic, nitric, and chromic acids) to macerate, and fuchsin to stain his *Hydra* preparations, was of the opinion that coelenterates possessed "neuromuscle cells" or "epitheliomuscle cells", which he described as epithelial cells that could carry a sensory hair and whose basal part consisted of a contractile fibre. According to him, phylogenetically these cells represented the first nervous system, with the contractile fibres giving rise to muscle cells and the rest to sensory nerve cells. Hertwig and Hertwig (1878) criticized this hypothesis, pointing out that specialization had to be the result of specialization of the cell as a whole and not of its parts. Since they had encountered cell bodies of coelenterate sensory nerve cells at various depths of the epithelium, they suggested that ganglion cells were derived from epitheliosensory cells whose cell bodies had come to lie beneath the epithelial cells. The formation of the nervous system could have come about by multiplication of the ganglion cells, and its associative capabilities could be accounted for by their communicating processes.

One focus of attempts to localize sensory structures and nerve cells continued to be the tentaculocysts. In their monograph on the nervous system of medusae, the brothers Hertwig (1878) also reviewed the various functions attributed to the tentaculocyst: Müller (1779-1784) interpreted them as organs of excretion, Rosenthal (1825) as mucous secreting organs, Eschscholz (1829) as livers, and Ehrenberg (1836) as sensory organs and their pigment spots as eyes (thereby changing his previous view,

that they were part of the male reproductive apparatus). Kölliker (1843) agreed with Ehrenberg that the pigment spots were eyes, but thought the tentaculocysts were auditory organs. Although Gegenbaur (1856) did not assign a specific function to the tentaculocysts, he counted them amongst the sensory organs but doubted that they had an auditory function. For Gegenbaur, who introduced the terms 'Craspedota' (Hydromedusae) and 'Acraspeda' (Scyphomedusae), which denoted the presence or absence of a true velum, the tentaculocysts were often more useful in the determination of the systematic position of a medusa than the body form or the arrangement of the tentacles. Agassiz (1862) viewed the tentaculocysts as composite eyes, not because of the pigment spots, but because of the accumulation of crystals in the tip of the tentaculocyst, and thought that they were modified tentacles.

Hertwig and Hertwig (1878) themselves contributed detailed examinations of the tentaculocysts of *Aurelia*, *Phacellophora* and *Pelagia*, which they collected during a stay in Messina, Sicily. Unfortunately, they were only able to obtain one specimen each of *Aurelia* and *Phacellophora*. *Pelagia*, like *Aurelia*, possesses eight tentaculocysts, while *Phacellophora* has sixteen. The Hertwigs described the striated circular muscle of the disc, but concentrated their descriptions on modifications in the margins of the three species, *e.g.*, the lappets, tentaculocysts, and gastric canals. A large part of the Hertwigs' results deals with the remainder of the ephyral lappets, which they referred to as sensory lappets. In their view, the latter represented typical, recurring structures which therefore had to be the starting point for any comparison of sensory structures between *Pelagia*, *Phacellophora* and *Aurelia*. Thus, they described in detail their development, relation to the tentaculocyst, and dimensions. In all three species, the lappets become greatly reduced in size compared to the rest of the body, due to disproportionate growth, and this reduction is most pronounced in *Aurelia*. Although this is also the case in Pacific *Aurelia*, their lappets are more prominent than

the ones in the drawings provided by the Hertwigs, to whom *Aurelia's* lappets appeared to be comparatively rudimentary organs.

As far as the tentaculocysts were concerned, the Hertwigs felt unable to attribute a specific sensory function to them. They did, however, identify the statocysts as continuations of the endodermal canals, [which extend into the tentaculocysts], and the pigment spots of *Aurelia* as ocelli. They described the tentaculocysts as sensory bodies whose outer epithelium consisted of a single layer of flat cells above the statocysts, and of ciliated cylindrical cells at their base. In the Hertwigs' view, the ectodermal swellings at the bases of the tentaculocysts represented the central portion of the nervous system, which consisted of sensory cells and a layer of "nerve fibrils". The sensory cells consisted of the cells of the ocelli as well as of touch cells, amongst which they considered including nematocysts. The "nerve fibrils" were of varying lengths and extended along the basis of the epithelium. Since they did not observe any ganglion cells, they concluded that the nerve fibre layer was composed solely of epithelial [sensory?] cell processes, which were either unbranched, bipolar, or had several secondary branches. The endodermal layer consisted of narrow cylindrical cells which also possessed cilia.

The Hertwigs also had the opportunity to examine an ephyra of *Pelagia noctiluca* and stated that its tentaculocysts showed only minor differences to those of the adult. They did, however, find that their bases were filled solidly by masses of endodermal cells. [I am not sure whether this is indeed true. However, this is not the case in the ephyral tentaculocysts of either *Pelagia colorata* or *Aurelia aurita*, which are hollow.] The Hertwigs did not find any "commissures" between the tentaculocysts, nor did they make any observation on the peripheral portion of the nervous system, although they did not doubt the presence of ganglion cells in the subumbrella.

Schäfer's (1878) attempt to find anatomical proof for the existence of nervous structures in medusae was based on examinations of specimens that were either fresh,

treated with "osmic acid", or stained with gold chloride. Unfortunately, he does not provide any details regarding his staining methods. However, according to his descriptions and drawings, the nerve cells and fibres were stained a deep violet, the nuclei remained unstained, and he observed some shrinkage of the tissue, indicating that he did not fix the tissue prior to staining (Lee and Mayer, 1907). In pre-fixed tissue the nuclei are darkly stained with gold chloride, the fibres are black, and shrinkage is less pronounced (Lee and Mayer, 1907). Some of Schäfer's drawings are based on sections, but the most striking ones are from whole mounts and osmium-treated tissue. Since the latter lacked the "distinctiveness of colouration", Schäfer did not stain it, and since he mentioned "teased tissue" and his drawings of the "osmic acid preparations" depict isolated neurons, he may have used osmium for maceration rather than fixation purposes; nevertheless, it does account for the reduced shrinkage in those preparations. [Osmium tetroxide, either alone or together with acetic acid, has been used for the dissociation of muscle tissue (Humason, 1972), and was used in maceration procedures at the time (Lee and Mayer, 1907).]

Schäfer divided the nervous system of *Aurelia* into the marginal bodies or tentaculocysts, the "nerve-epithelium" in their vicinity, and the subumbrellar nervous plexus. The latter consisted of an interlacement of nerve fibres associated with the muscular sheet, lying between the muscles and the ectodermal cells. He worked on *Aurelia* because of its availability, but also made some observations on *Chrysaora*. In both, he observed bipolar ganglion cells of the subumbrellar plexus. The cells were 50 μ m in length and 15 μ m in diameter, and possessed a spherical or ovoid nucleus. Their fibres covered the entire muscular sheet, but were least abundant near the margin. He traced some fibres into the tentacles, but did not see any associated with the gastric canals. Although he observed some fibres converging towards the tentaculocysts and the "nerve-epithelium", he could not follow any fibre into either. Schäfer stressed that the fibres were not structurally continuous with any other fibre,

that each fibre belonged to a bipolar (or rarely a tripolar) nerve cell, and that the fibres were rarely branched. [It is to his credit that he was adamant about the individuality of the neurons, since he clearly expected to find anastomoses.] The fibres often ran parallel to each other and sometimes intertwined, and most of the fibres ended in a gradual tapering. Schäfer concluded that the function of the subumbrellar plexus was to cause the simultaneous contraction of the bell muscles, and that somehow nervous impulses were transmitted from fibre to fibre. Further, because of the proximity of the fibres and the ectodermal epithelial cells, any exterior stimulus would rapidly spread through the plexus to the muscles.

Schäfer's description of the ectodermal covering of the tentaculocyst paralleled that of Hertwig and Hertwig (1878), with whose work he was familiar. He, too, observed the pigmented cells, but stated that definite proof that they performed the function of an ocellus was lacking. He found the epithelial cell fibres that passed through the sub-epithelial stratum to be less defined than those of the subumbrellar surface and to have "far less decidedly the character of nerve fibres". He also expressed his surprise that although there were characteristic nerve cells in the subumbrellar plexus, no definite nerve cells were present in the tentaculocyst, which he, too, identified as the representative not only of the central nervous system but also of sense organs. Interestingly, he saw some endodermal cells whose ends tapered into processes and questioned whether they could be nerve fibres [indeed they could!]. Some of the fibres bridged across the mesogloea from the endodermal to the ectodermal side. He regarded all the fibres, regardless of their origin, as forming a rudimentary nervous system, which, in a phylogenetic sense, represented the beginnings of a "central nervous apparatus". Of the nervous and muscular tissue of the subumbrella he noted that "it is interesting to observe, even so low down in the metazoic scale as the medusae, that the textures, which in the higher animals are generally looked upon as the most highly differentiated, should have already attained a degree of structural

complexity and functional activity in many respects scarcely inferior to the nervous and muscular tissues of the vertebrates". [True, but quite an amazing statement, considering that just two years prior, Romanes (1876) had stated that it was safest to assume that they did not possess a nervous system.]

When Romanes (1876, 1877/78, 1880) started out his research on *Aurelia*, he was not aware of any histological evidence as to the presence of a nervous system. Romanes undertook a series of physiological experiments on the locomotor system of medusae, including *Aurelia aurita* and *Cyanea capillata*, which he obtained from the east coast of Scotland. His (1876) experiments included the removal of tentaculocysts and the excision of tissues to determine their impact on swimming, and the addition of various chemicals to the water. He found that the marginal supply of locomotor centers was concentrated in the tentaculocysts, and that the removal of all but one tentaculocyst still insured the continuation of the swimming beat. He was certain that the margin represented the principal seat of spontaneity, but was unsure if it was the exclusive seat. The oral arms continued to move after removal of the tentaculocysts and even after they were completely severed from the body. Therefore, Romanes removed the oral arms (in addition to all but one of the tentaculocysts) in experiments on dissected animals. In one such experiment, he made the remaining tentaculocyst the point of origin of a spiral section of the disc, resulting in a long strip of tissue. In another experiment, he cut alternating radial sections both from the margin and the center of the animal, resulting in interdigitating strips of tissue. In a third experiment, he dissected an animal into two circles that were only connected by a narrow (about 3 mm wide) bridge of tissue, which was on the opposite side of the tentaculocyst. In all three cases, the contractions continued. When he severed the bridge connecting the two circles in the third experiment, the movements of the inner circle stopped, while they continued in the outer circle as before. Also, pressure applied to any part of a contractile strip blocked the contractions, sometimes permanently, depending on the

duration of the exerted pressure. Romanes explained the results by suggesting that a "more or less intimate plexus" of "lines of discharge" mediated the spontaneous impulses originating in the margin to the contractile tissue, and that these lines therefore were functionally nerves.

In order to compare the specialized marginal tissue with nervous tissue in general, Romanes examined the effects of a number of chemicals on the locomotor system. He found that 1.3 to 2 minutes after the addition of chloroform to the water, and 2 minutes after it was placed in a "strong" sea-water solution of morphine, *Aurelia aurita* ceased its movements, but recovered fully in normal sea-water. The pulsations of *Cyanea capillata* became irregular after about ten minutes in a "weak" solution of strychnine, turning into spasms followed by periods of absolute quiescence. The convulsions could last as long as five or six minutes, while the periods of quiescence lasted no longer than 40 seconds. Romanes also exposed half of a medusa for 30 minutes to a 1:2500 solution of curare, while the other half remained in normal sea-water. The contractions in the exposed half stopped, while the unexposed half continued to beat. When the exposed half was mechanically stimulated with a pair of forceps, it remained motionless. The animal recovered fully in normal sea-water. [Given the mode of action of curare, one could deduce that acetylcholine is present as a neurotransmitter. However, other researchers have failed to confirm Romanes' results, even when using high concentrations of curare (Passano, 1982), and Bullock and Nachmansohn (1942) could not find any appreciable acetylcholine esterase activity, which is required for acetylcholine function.] Romanes repeated the experiments on strips of contractile tissue from *Aurelia*, parts of which he exposed to the various chemicals. In all cases, the contractions stopped where the strips contacted the poisoned water, but resumed when the tissue was restored to sea-water. The contractions were also interrupted when Romanes injected any of the chemicals into the openings of gastric canals created by a circumferential cut. Since endodermal canals

terminate in the tentaculocysts, Romanes speculated that this way of administering the chemicals could be more direct than any other way, but cautioned that the paralyzing effect of the poison could be due to its influence on the contractile tissue, rather than on the center of discharge. Romanes noted that tentaculocysts that had been injected with morphine or chloroform recovered overnight, while they returned to their normal state in a matter of a few hours after the injection of alcohol, which at first caused more potent and more frequent contractions that gradually subsided to total quiescence.

It is not quite clear where exactly Romanes thought "the center of spontaneity" was located, for he attributed it to the "marginal bodies", by which he could mean all of the tentaculocysts, but also stated that he was able to satisfy himself that the whole spontaneity "appeared to be exclusively lodged in the minute sac of crystals", by which he probably meant the statocyst. However, he arrived at his conclusion not by comparing the effects of removing the "sac of crystals" versus removing the whole of the tentaculocyst, but instead by comparing the effects on contractions of removing the "sac of crystals" versus the "curious wing-like appendages". Exactly what he meant by the latter is not quite apparent in his 1876 paper, neither from the description nor from the drawing provided, of which he conceded, but did not elaborate, that "in some detail the drawing is not quite accurate". In 1877, after he had repeated some of his previous experiments and carried out some new ones, he defined the wing-like appendages as "minute diverticula of the nutritive tube system", and stated that he had not been able to find any physiological evidence for the presence of ganglionic matter in those zones. Reemphasizing that the "whole spontaneity appeared to be exclusively lodged in the minute sac of crystals", he nevertheless now added "in (or about) the minute sac". He now referred to the locomotor center as a ganglion, because of "more recent histological observations".

Romanes (1877) determined that any mutilation of the animals influenced both the regularity and the frequency of the swimming beat, and suggested limiting

circumferential cuts to the cellular part of the subumbrella while leaving the mesogloea intact. He also found that an increase in water temperature led to irregular contractions and frequent pauses between beats, which was irreversible if the temperature was high enough (about 27°C for animals originally kept at about 4.5°C). The contractions could be restored, however, if the animals were subjected to cold water. Surprisingly, Romanes found that even animals that had been frozen solid in sea water recovered after being returned to their normal temperature, and that the recovery took from a few minutes to about 30 minutes.

In addition to mechanical, chemical, and thermal stimulation, Romanes (1877) also investigated the influence of electrical stimulation on the swimming beat. Of the conditions that modified the "period of latent stimulation" [response latency] in *Aurelia*, he considered the most important influence, from a physiological point of view, to be the summation of stimuli, which he referred to as the "staircase". The summation of stimuli not only led to a decreased latency, but also to an increased amplitude of the contractions.

In addition to the wave of contractions, which proceeded at 45.75 cm/s, Romanes also observed another kind of wave, that of tentacular contraction, which proceeded at 22.86 cm/s. The stimuli that initiated a tentacular wave were "too gentle to start a contractile wave", and were most successful in "perfectly fresh and vigorous" animals. Since both kind of waves continued even after the tissue had been subjected to severe forms of sectioning, Romanes was "driven upon the theory of a nerve-plexus whose constituent elements are capable of vicarious action in almost any degree".

In 1880, Romanes investigated the effect of stimulation on the persistency of the rhythm of the swimming beat. He found that with electrical stimulation, the rhythm of a piece of *Aurelia* whose tentaculocyst had been removed remained regular for over one hour. He was also able to produce an artificial rhythm through constant mechanical and chemical stimulation, but found that it was less sustained than the rhythm produced

by electrical stimulation. Stimulation with light resulted in increased activity in intact pieces of *Aurelia*, whereas it had no effect on pieces without tentaculocysts. Finally, Romanes determined that when he cut the subumbrellar surface of *Aurelia* in a manner that left the mesogloea intact, regeneration of the severed tissue occurred in four to eight hours.

Eimer (1878) carried out a number of experiments very similar to those of Romanes, but also added some morphological studies. Eimer's studies were meticulous, he repeated his experiments often and took great care to recreate the same conditions for the repetitions. However, one cannot help but get the sense that Eimer did not quite have the same grasp on the nervous system of *Aurelia* as Romanes did. Romanes and Eimer had been in disagreement on some of the results of their studies, *e.g.* the precise seat of the nervous centers in *Aurelia aurita* and the strength of the contractions in the latter after removal of the tentaculocysts, and also on who could lay claim on having made some of the discoveries first. By 1880, their disagreements, which had been partly due to misunderstanding compounded by the different languages, were settled. Eimer had started his investigations on *Aurelia* and *Cyanea* in 1873 on the Baltic Sea, and complemented them in 1876 and 1877 with studies on *Pelagia* and *Rhizostoma* in Naples. He emphasized that Romanes had not begun his experiments until 1874, but that, curiously, their studies were in conformity with one another, although each had been unaware of the other's work at first.

Eimer was motivated by the question of the beginnings of the nervous system in the animal kingdom. His goal was to find out where structures could be found first that could, without doubt, be considered nervous systems, and whether a comparison between them and other systems could shed light on the connection between ontogeny and phylogeny [he was a contemporary of Haeckel]. He was concerned with the question of whether the medusae were capable of voluntary movements, but was quick to point out that, for him, the only difference between 'voluntary' and 'involuntary'

was due to the fact that a 'voluntary' activity required that impressions were collected and stored in a common organ (brain) and had the potential to interact. He considered the involuntary contractions of the medusae to be "pure feeding movements, *i.e.* they serve foremost in breathing, but also carry food into the stomach". The rhythm of these contractions was so regular that one could count them "without looking at the animal", and the number of the former was inversely proportional to the size of the animal. Eimer observed that the contractions originated from the immediate vicinity of the tentaculocysts. This area, then, controlled all of the vegetative activities of the animal. Although ciliary movements were involved in feeding, Eimer had determined that water currents created by contractions of the bell were necessary for food uptake, which did not occur in motionless animals. [He was wrong. When presented with an abundant food source, such as *Artemia*, adult *Aurelia* cease swimming and secrete a large amount of mucus. *Artemia* entangled in the mucus (and possibly immobilized by discharged nematocysts) continue to be taken up.] He deduced that sperm uptake, too, depended on the female's ability to create these water currents. The movements became voluntary as soon as the animal changed positions, and Eimer thought that the medusa was capable of initiating a contraction, at will, from any segment [antimere, *i.e.* an eighth of an animal] that possessed a tentaculocyst. Since the animal could determine the sequence of contractions of the segments, it could steer itself in various directions.

In order to determine the extent of the control of the nervous centers, Eimer (1878) undertook a number of experiments, some of which he carried out from a boat. He then either observed the animals from the boat, or kept them in the water in boxes. In one set of experiments, he removed all, or all but one of the tentaculocysts in specimens of *Aurelia*, and found that animals with just one tentaculocyst intact remained for days as active as unoperated animals. Animals without any tentaculocyst could still show some contractions, which were frequently irregular and weak, but

gradually the animals disintegrated. Any sections of *Aurelia*, whether they were halves, quarters, or even antimeres, continued to contract, and remained alive for days. When Eimer made gradually larger radial cuts in an animal, resulting in an antimere that was still connected to the rest of the animal, the contractions of all parts remained synchronous until the cuts approached the gonadal tissue (6.5 to 8 mm distance from the gonads in an animal of 13 cm diameter). Likewise, a strip of tissue connecting two halves of an animal had to be less than 4 mm wide in order for the synchronous contractions to stop. Eimer was thus able to determine that the synchrony of the contractions did not arise from a marginal nerve ring. Neither did they arise from a nerve ring surrounding the gonads, since radial sections from the center of the medusa towards the periphery did not disrupt the contractions unless the sections almost reached the margin. These and experiments similar to those of Romanes (1876), using circumferential and radial cuts in specimens of *Aurelia* and *Cyanea*, convinced Eimer that the peripheral nervous system of the medusae had to consist of a plexus of nerve fibres, and that nervous centers existed in the areas of the tentaculocysts. As far as the latter were concerned, Eimer disagreed with Romanes (1876) that the removal of the statocysts alone could account for the permanent disruption of the movements. He found that in both *Cassiopea* and *Rhizostoma*, paralysis due to removal of the statocysts was only temporary as long as the rest of the tentaculocysts remained intact. Eimer did concede, though, that the viability of the preparations was greatly influenced by the age and temperature of the water in which the animals were maintained, and that differences in any of the results could also be due to differences in the "nervous constitution" of the animals, differences in the outside temperature, and to whether or not the animals had been feeding. Eimer repeatedly observed that even after the removal of the entire tentaculocyst from antimeres of *Aurelia*, rhythmical contractions continued, and that the latter stopped only after he also removed the "contractile zones". There had been some, in Romanes' (1878) terms, "misapprehension" as to the

exact definition and location of the latter until, through correspondence between Eimer and Romanes, it became clear that the area in question pertained to the microscopical vicinity of the statocyst. The latter definition still appears somewhat vague and surprising, especially since Eimer's descriptions of the operations stated that, in order to stop the contractions completely, he had to remove not only the tentaculocyst, but also some of the surrounding subumbrellar tissue, to which sometimes mesogloea adhered. Later on in his paper, Eimer even stated that the basic composition of the contractile zone was that of nerve cells and nerve fibres, and of "muscle elements of the subumbrella". Eimer also mentioned that he observed some animals and segments of animals which recovered from the removal of the tentaculocysts so that, after some time of paralysis, the rhythmical contractions returned, sometimes as soon as three days after the operation. Eimer tried to explain these results by assuming that, under optimal conditions, groups of subumbrellar nerve cells would assume the role of locomotor centers. Eimer stressed, however, that the recovery was only of short duration, and that the animals died shortly thereafter. It should also be noted that Eimer's prerequisite for 'rhythmical' appeared to have been met as soon as a number of consecutive contractions occurred.

Eimer also reviewed earlier work on rhythmicity and other aspects of medusan biology. In 1778, Macri reported that separated pieces of *Rhizostoma* continued to live. Gäde (1816) made the same observation on *Aurelia aurita*, but did not believe that it was able to regenerate. Eysenhardt (1821) speculated that the contractions of pieces of *Rhizostoma*, which were stronger when stimulated mechanically, were due to muscle tissue. Spallanzani (1826), too, determined that muscle tissue was responsible for the contractions of medusae, but was undecided on whether the movements were voluntary or not. Mettenheimer (1862) on the other hand, was convinced that the contractions in *Aurelia* were voluntary, since they could be weaker or more powerful, thereby regulating the speed of the locomotion, and medusae in an aquarium could

swim in one direction for a long time, as if they intended to approach a particular object. Although Mettenheimer was unsuccessful in finding any ganglia, he assumed that they were present in the margin and also existed in the center of the disc, in order to regulate, coordinate, and integrate the movements and make them rhythmical.

In contrast to Romanes, Eimer (1878) also undertook some histological studies. His results on the gross morphological structure of the tentaculocysts, which he thought were modified tentacles, were similar to those of the Hertwigs (1878). In order to determine the extent and the elements of the nervous system, he examined whole mounts, macerated preparations, and sections. Since his attempts to stain the neurons with gold chloride failed, he stained his specimens with carmine [this does not necessarily reflect on his capabilities; apparently, the gold chloride technique can be temperamental]. According to Eimer, the central components of the nervous system consisted of nerve and ciliated sensory cells associated with the tentaculocysts and the tissue immediately surrounding it. On the oral surface, the peripheral nervous system consisted of nerve cells associated with the subumbrellar muscle, sensory and 'neuromuscle cells', *i.e.* cells that possessed both nerve and muscle fibres. The tentacles, too, possessed sensory cells, and nerve cells were also present on the aboral surface surrounding the lappets. He observed the cup-shaped appearance of the sensory cells, the dichotomous branching of, and the varicosities in the nerve fibres, and reported some anastomoses between the latter (which he considered to be an advanced trait; in most of his drawings, nerve cells are represented as individual cells). Eimer thought that the subumbrellar plexus of nerve cells collected impulses from the nervous portion of the 'neuromuscle cells' and spread them throughout the contractile surface, and that both cell types had an "original anatomical connection". Thus, nerve cells could have arisen through specialization of the 'neuromuscle cells' [he was also a contemporary of Kleinenberg]. He also speculated that nerve cells could be differentiated amoeboid cells, since he had observed the latter in the mesogloea, and

since both cell types were of ectodermal origin, as were the muscle cells [the amoeboid cells would thus parallel hydrozoan interstitial cells, which can differentiate into different cell types. This theory is still entertained today.] In the mesogloea of *Aurelia*, he observed chains of amoeboid cells, and assumed that they could form nerve cells when needed and transmit signals. If they were needed frequently, they could gradually transform and become permanent nerve cells. He also thought that the fibrils which he found in the mesogloea and which he thought to be of ectodermal origin, could have a nervous function. Sensory organs developed through gradual morphological differentiation of the ectodermal tissue elements. The higher developed the sensory organ, the more it was localized, and the sharper the boundaries between it and the surrounding tissue. Thus, sensory organs developed from sensory epithelium, but, except for the tentaculocyst, Eimer could not distinguish any boundaries between accumulations of sensory cells. Because of the lack of morphological differentiation of the sensory epithelium covering the surface of the animal, Eimer stressed that he could only guess that it provided the animal with the senses of touch, taste and perhaps smell.

von Lendenfeld (1882) undertook extensive histological examinations of several *Cyanea* species of the South Sea. Though some of his observations are quite astute, *e.g.* the ones on nematocysts, his drawings make it quite clear that 'contacts' between cells of the nervous system consisted of extensive anastomoses, for not only did he draw the latter between ganglion cells in the epithelium, but also between sensory and underlying ganglion cells. He compared his results often to those of Eimer (1878) [who also described anastomoses]. von Lendenfeld found small round cells in the mesogloea which were similar to those described by Eimer (1878). The cells, which frequently occurred in pairs, were more abundant close to the ectoderm than in the middle of the mesogloea, and were absent close to the margin. von Lendenfeld disagreed with Eimer as to the nature of the mesogloeaal fibres, of which he observed two types, smooth and varicose ones. He was convinced that the smooth fibres

belonged to connective tissue, but conceded that the varicose fibres could perhaps belong to nervous elements. However, this could not be proven since he did not find a connection between the fibres and ganglion cells.

Distributed over the entire surface of the umbrella, von Lendenfeld (1882) found sensory cells and nematocysts among supportive epithelial cells. The processes of the sensory cells, which could reach a "considerable" length, as well as the nematocysts frequently made contact with subepithelial ganglion cells. He doubted that the nematocysts were cnidocil-bearing sensory cells because of the shortness of the cnidocil, and thought that the direction of nervous transmission was towards the nematocysts. He tested his hypothesis by applying concentrated acetic acid to the free end of isolated tentacles that were covered by a cover glass. The nematocysts discharged simultaneously along the entire length of the tentacles, before the acid was completely mixed with the sea-water, while adjacent tentacles only discharged their nematocysts when the acid reached them. He therefore deduced that external stimuli caused nematocyst discharge directly and mechanically as well as indirectly through nervous signals [a century later, there are still two schools of thought on the control of nematocyst discharge (Westfall, 1989), and even with the EM, there is evidence only for *presumed* neuronematocyte synapses.] Nematocysts also occurred in clusters, which also contained sensory cells of 15-20 μm length.

The ganglion cells of the subepithelial layer had a diameter of 10 μm , and their processes ran tangential to the body surface. Sensory, supportive, ganglion and muscle cells as well as nematocysts were also found in the tentacles and the oral arms. The muscle-free areas of the subumbrellar margin were characterized by a lack of nerve cells. Here, sensory cells comprised 0.5 % and glandular cells 1.5% of the number of epithelial cells. von Lendenfeld found ganglion cells of varying sizes closely associated with the muscle layer, and thought it probable that they were in nervous contact with the sensory epithelium of the tentaculocyst. He was unable to find any points of

contact between the ganglion and muscle cells, and did not believe that nerve and muscle fibres were continuous. The endodermal cells consisted of ciliated cells with a height of 9 μm , and evenly distributed glandular cells which comprised 5% of the endodermal cell population. No sensory or ganglion cells were present in the endodermal layer, but he found nematocysts in the gastric filaments, whose discharge therefore had to be triggered purely mechanically [he was wrong. There are nervous elements in the endoderm]. In the tentaculocysts, von Lendenfeld observed fibres as well as sensory and multipolar ganglion cells in the ectoderm, while glandular cells were missing in the endoderm.

von Lendenfeld also examined ephyrae [one of the few to do so], the smallest of which measured 5 mm in diameter. In their tentaculocysts, the distinction between sensory and supportive cells was not quite as clear as in the adult, and the basal portion of the tentaculocysts consisted of supportive rather than sensory epithelium. As far as the function of the sensory cells was concerned, von Lendenfeld speculated that the latter represented sensors for changes in pressure, temperature, and chemical composition of the surrounding medium. Another difference between the ephyra and the adult was found in a bundle of fibres which extended from the base of the tentaculocyst towards the center of the subumbrella. In the ephyra, the fibres had more bends and were situated, together with their cell bodies, in the epithelium, whereas in the adult, the fibre bundle was smooth and was located in the subepithelial layer [there is no such bundle in *Aurelia*]. Finally, while the margin of the manubrium of the ephyra demarcated the boundary between endoderm and ectoderm, in the adult the ectoderm extended to the inside of the oral arms [the same is true for *Aurelia*].

In 1895, Hesse's studies on the nervous system of *Rhizostoma* confirmed the main observations of previous researchers on other scyphomedusae (Eimer, 1878; von Lendenfeld, 1882). He, too, observed the structure and components of the sensory epithelium of the tentaculocysts, and often saw fibres extending into the mesogloea.

Peripherally, Hesse noticed a plexus of numerous, mostly bipolar cells on the subumbrellar surface. The fibres frequently approached and ran parallel to one another, and he could follow some fibres for up to 6 mm. Their diameter was $7.2\mu\text{m}$, and that of the cell bodies measured $18\mu\text{m}$. The ganglion cells and their processes passed parallel to the surface of the animal, below the layer of nuclei of the supportive cells, and, some were almost parallel to the radial endodermal canals, others proceeded in a more circular fashion. Hesse, too, operated on some specimens by severing the tentaculocysts or the tissue between them, and thought that the tentaculocysts communicated with one another through a number of nerve fibres.

Bethe (1908,1909) studied the effect of changes in exterior salt concentrations on the rhythmical pulsations of medusae, and attempted to determine whether the muscle, the nerve plexus, or the tentaculocysts were primarily affected. He found that the swimming beats of octants of *Rhizostoma* did not change in 80% sea water diluted with distilled water, while a 75% solution decreased the frequency of the beats after 20-30 min. and arrested swimming completely after 6-8 h. The effects in 50% sea water were even more pronounced. The specimens did recover, however, in normal sea water. Calcium-free artificial sea water quickly caused complete, but reversible paralysis. The addition of small amounts of calcium salts resulted in prolonged and stronger pulsations, and postponed death, whereas the addition of magnesium salts had a paralyzing effect [the latter had been introduced as anaesthetizing agents in 1891 (Lee and Mayer, 1907)]. By exposing the tentaculocysts alone while the rest of the preparations remained in normal sea water, Bethe (1909) could achieve the same results more quickly and with weaker salt solutions. Furthermore, by exposing only one tentaculocyst in an intact medusa, he could increase the swimming beat, which returned to the previous level after the removal of that tentaculocyst. He therefore concluded that the swimming rhythm was under the control of that tentaculocyst which initiated the highest number of impulses. If the number decreased below that of any of the other

tentaculocysts, the latter would take over, so that even the paralysis of one tentaculocyst was of no consequence for the whole animal.

Mayer (1916) phrased Bethe's statement thus: of the marginal sense organs, "one only, the one which for the moment works the fastest, controls the rate of pulsation". When he grafted a small and a large *Cassiopea* together so that the subumbrellas made contact, the two medusae contracted in unison at the rate of the small medusa, *i.e.* more rapidly than the large medusa alone. Conversely, mechanical stimulation of the large medusa in the graft increased the rate of contraction. Mayer could also produce contraction waves in tissues isolated from the central nervous system in a number of medusae, including *Aurelia* and *Cyanea*. When he removed all of the tentaculocysts and the oral arms of a *Cassiopea* and cut a ring of muscle tissue from the disc, a local stimulus caused the spread of a contraction wave in either direction. When he blocked the wave from going in one direction, it continued in the other direction and could go around the ring for days, thus producing an entrapped wave. He found that, although the marginal sense organs were most sensitive to stimuli, even a ring of tissue which lacked sense organs but was pulsating by means of an entrapped wave responded to local stimuli by augmenting the muscular contractions. Muscular activity in *Cassiopea* was stimulated by the combination of sodium, calcium and potassium, but inhibited by magnesium. He also found that the muscles were much more affected by temperature, salinity, or excess or deficiency of ions than the nervous system. For example, muscular movements were all but extinguished in an animal heated from 29°C to 38°C, while nervous conduction continued at 1.5 times the former rate until the temperature reached close to 42°C.

Motivated by the "intimate" relationship between the muscle and nervous systems, Krasinska (1914) shifted the emphasis of her histological studies from the nervous system to the medusan muscles. Hers were the first detailed observations of the muscle striations, the epithelial character of the muscle cells, and the relationship

between muscle somata and their contractile tails. While medusan muscle cells are found in the lattice of epithelial cells, the mesogloea underlying the muscle cells is frequently folded, thus providing an increased surface area. These mesogloea folds had either not been mentioned previously (Eimer, 1878) or, as Hertwig and Hertwig (1880) had assumed, were connected to the formation of a separate, "mesodermal" muscle layer beneath the epithelium. According to the Hertwigs, increased muscle activity required an increase in muscle volume and therefore folds in the mesogloea. Because of these folds, the muscle cells were positioned beneath the epithelial cells, where they formed a separate muscle layer. Krasinska questioned this causal relationship and illustrated that muscle cells can remain epithelial despite prominent mesogloea folds, which is the case, for example, in the subumbrella of *Pelagia*. As to the connection between muscle cells and fibres, Krasinska entertained two possibilities: since a number of muscle fibres pass below any given epithelial cell in the muscular areas of the medusa, the fibres could belong to a syncytium, formed by the basal parts of the epithelial cells, or the musculature could be composed of individual muscle cells, so that each fibre would project from its own cell body. In the latter case, a single muscle cell could produce either one or several fibres. In sections, the basal part of the subumbrella ectoderm of *Pelagia* appeared to Krasinska to be syncytial, but in preparations of macerated tissue, she obtained individual muscle cells, which possessed one fibre each. She cautioned that despite the appearance of the sections, a syncytium need not be present.

Krasinska (1914) also examined the nerve plexus and found large and small ganglion cells in the subumbrella and tentacles, and sensory cells in the subumbrella, but not the tentacles, of *Pelagia*. (She did, however, predict the existence of sensory cells in the tentacles based on the speed of their reactions to stimuli). Although she was unable to prove the innervation of the muscle cells with her own observations, Krasinska regarded the latter as a fact, which she felt was supported by the high

number of ganglion cells in the area of the musculature. She believed the large cells to have a motor function, and postulated the existence of a second, sensory nerve net, since the small ganglion cells made contact with the sensory cells. In contrast to Bethe's (1903) findings in *Rhizostoma*, Krasinska did not observe any direct connection between the cells in *Pelagia*, and therefore doubted that a true nerve net was present. She also disagreed with Kleinenberg's (1872) postulation, *i.e.* that epitheliomuscle cells could be stimulated directly and transmit stimuli to muscle fibres, a view that had been upheld by Claus (1878). To Krasinska, a direct stimulation did not seem to be required, since the functioning of the medusan musculature could be explained sufficiently by the fact that the muscle cells were innervated, and by the ability of the peripheral nervous system to communicate external stimuli to the muscle cells.

As far as the muscle striations are concerned, Eimer (1878) had described striations in the myofibrils of muscle cells, but Schneider (1892) denied a true striation, stating that the muscle fibres resembled a string of pearls with alternating thicker and thinner parts, but without any structural differences. Krasinska attributed Schneider's observations to poorly macerated tissue preparations, and in turn was able to distinguish between dark and light bands, the Z-disc, and the H-band (a light band in the middle of the dark band). She also provided measurements of the dimensions of the fibrils (2 μm width), the repetitions of the striations (every 1.6 μm), and of the bands (in proportion to one another). She saw fibres with two fibrils, and speculated that the tapered ends of the fibres contained two, but the middle and thicker part possibly several fibrils.

Krasinska also examined the smooth muscle cells of the oral arms of *Pelagia*, which consisted of true epitheliomuscle cells. Each cell produced one very long and narrow fibre that appeared completely homogenous. In the tentacles, on the other hand, the muscle cells were arranged in "muscle folds" in the massive mesogloea. While mesogloea folds of the subumbrella reach into the ectodermal layer, in the tentacles the mesogloea is penetrated by folds whose walls provide an increased surface

area for the attachment of the longitudinal muscle fibres. The centres of the folds contain muscle cell bodies and nerve cells. In cross-section, the tentacles are oval and appear bilaterally symmetrical due to the distribution of the folds (the depths of the folds decrease with distance from the single median fold). Krasinska counted 21-25 folds in the proximity of the margin, but noted that their numbers diminished toward the tip of the tentacle. Except for the median fold and its immediate neighbours, the folds are divided into several segments by longitudinal indentations of the mesogloea. Complete indentations result in the formation of "muscle tubes" which are totally separated from one another and from the epithelium, while the segments of a fold remain connected with incomplete indentations. Similar "muscle tubes" had been described by Claus (1878) in the tentacles of *Charybdea marsupialis*, and had prompted the Hertwigs (1879) to apply the term "mesodermal" to their position in the mesogloea.

Thus, by the turn of the century, the life cycle of scyphozoans was understood, although details of the developmental changes, especially those occurring during strobilation, were either in dispute or not known. The elements of the nervous system, and that of the tentaculocysts in particular, had been investigated both physiologically and histologically, and it was generally agreed that the nervous centers in the tentaculocysts communicated both with one another and with the peripheral nerve plexus.

Bozler (1927) attempted to determine how this communication took place, *i.e.* whether the nerve cells were in contact or continuous with one another, and whether more than one nerve plexus existed. The latter question arose when Bozler (1926a,b) tried to analyse the behaviours he had observed in *Pelagia*, *Rhizostoma* and *Cotylorhiza* in addition to the fast swimming contraction. Tilting the animals resulted in compensatory movements, *i.e.* modified swimming movements, stimulation of the tentacles or the subumbrella led to an upwards movement of the oral arm closest to the stimulus, and isolated pieces of oral arms exhibited crawling movements. He also

observed slow contractions of the circular muscle that spread radially and only towards the periphery. In *Pelagia*, the slow contractions travelled at 1cm/s versus 24 cm/s for the swimming contractions.

For the swimming movement, Bozler (1926a) had determined that, of the components of the tentaculocyst, the ganglion alone was responsible. When he removed the one remaining tentaculocyst of an animal piece by piece, only the destruction of the ganglion permanently arrested swimming. Since the removal of the sensory epithelium only had a temporary effect by briefly decreasing the frequency of the contractions, Bozler concluded that sensory input was not required to maintain the beat. He proposed that instead the rhythm was due to impulses that were sent out rhythmically from the ganglion and carried by elements of a nerve net, and since the impulses travelled fast and the tentaculocysts regulated one another, synchronous beating was assured.

For the slow contractions, Bozler (1926b) proposed that a second nerve net was responsible in which impulses travelled at a slower speed, and since the contractions only spread in one direction, the synapses had to be polarized (irreciprocal) as opposed to the non-polarized fast nerve net. [Bozler did mention, however, that the polarization was not absolute, since it disappeared in animals that had been left in standing water for a day.] He further suggested that not all of the muscle fibres that contracted during swimming were involved in the slow contractions.

For the compensatory movements, Bozler (1926a,b) had shown that the tentaculocyst also acted as a statocyst. When he kept an animal in a fixed position, bending the statocyst downwards (towards the subumbrella) with a needle led to an increased beating frequency. The compensatory movements consisted of synchronous beats, but the circular muscle closest to the manipulated statocyst did not fully relax. Bozler proposed that a third nerve net was responsible that could slow down the muscle contractions locally.

As far as the movements of the oral arms are concerned, Bozler (1926b) refrained from offering a physiological explanation, but suggested that the fact that the muscles on the oral arms are smooth rather than striated was probably important.

Mainly on the basis of the proposed polarized synapses, but also because some of the behaviours could occur simultaneously, Bozler (1927) assumed that the neurons were physiologically separated, and that different, overlying nerve nets brought about the different responses. In order to demonstrate that the physiological separation was based on morphological discontinuity, he stained the neurons of *Rhizostoma* with methylene blue. He found both bipolar and multipolar neurons on the subumbrellar surface. The bipolars were associated with muscle, but also occurred in muscle-free areas, and based on their sizes, Bozler divided them into three categories, which he speculated were of physiological importance. The direction of the nerve fibres, which were always unbranched, was irregular, except for the vicinity of the tentaculocysts. There, the fibres ran parallel to one another and seemed to originate from the tentaculocysts. In other muscle-free areas, the direction of the fibres was predominantly circular. Bozler (1927) never found any fusion of the fibres, noting that they always remained separate, and emphatically denied the presence of anastomoses.

In the plexus of multipolar cells, the fibres branched dichotomously and were characterized by varicosities. The fibres often followed those of the bipolar cells closely, which Bozler assumed allowed for a diffuse transmission of impulses, and he speculated that a given multipolar cell made contact with only one of the three types of bipolar cells. He did not observe direct contact between nerve and muscle cells in his preparations. Finally, Bozler (1927) briefly mentioned a nerve plexus in the endoderm consisting of small bipolar cells, which also did not anastomose. He concluded that there was no evidence for the presence of a true, continuous nerve net, and that therefore the structure of the nervous system of coelenterates could not be used as the main argument against the neuron theory (Bethe, 1903).

In 1940, Bullock stated that properties of the coelenterate nervous system such as diffuse conduction and autonomy of fragments pointed to the existence of a nerve net, which was probably synaptic, since anatomical discontinuity had been reported. Moreover, these synapses had to be unpolarized, with conduction occurring in all directions. He thought that these "physiological" nerve nets "may exist side by side" with continuous or "anatomical" nerve nets, but that the latter were probably derived from and not ancestral to the former. However, he did not elaborate on whether or not he thought that the two nerve nets could coexist in coelenterates.

According to Bullock (1943), "one of the outstanding characteristics of the primitive nervous system....is the high degree of dependence on a process which Pantin named "facilitation" presumably taking place at the myo-neural junction and probably in certain regions at neuro-neural junctions (i.e., synapses)". Previous work had suggested that facilitation [in which each successive synaptic potential becomes larger than the last] occurred in medusae (Romanes, 1877), and sea anemones (Pantin, 1935), but Bullock (1943) was the first to employ carefully controlled single stimuli to study neuromuscular facilitation in scyphomedusae. [However, Bullock's preparation did not allow him to deliver stimuli to any single type of neuron, so that his demonstration was of 'absolute' rather than 'relative' facilitation, as was Pantin's.] He found that each successive stimulus delivered to a strip preparation of a demarginate medusa resulted in a contraction that was higher than the previous one, until a plateau was reached that could last for long periods. The optimal interval between stimuli was 2s and any adequate stimulus was maximal. Compared to sea anemones, facilitation developed more slowly, reached its maximum later, and the refractory period was longer in medusae. While excess calcium and potassium had no appreciable effect, excess magnesium depressed and eventually blocked facilitation (90% 0.88M MgSO₄ in sea water). Bullock also showed that the spontaneous swimming contractions initiated by the tentaculocysts exhibit facilitation, and that it, rather than control by the

tentaculocysts, determined the strength of the beats. He showed, thereby, the importance of facilitation for the swimming behaviour.

The Modern Era

Jellyfish research resumed after World War II with studies by Horridge (1954, 1956) on both neuroanatomy and neurophysiology. This work used electrical recordings for the first time. With the introduction of electron microscopy in the 1950's and the use of intracellular as well as extracellular recordings, the analysis of scyphozoan behaviour moved increasingly to the cellular level. This work has been reviewed in detail by Bullock and Horridge (1965), Passano (1982), and by Satterlie and Spencer (1987).

Modern findings regarding topics important to the present study will be referred to where appropriate in the text.

In 1953, Horridge published the first record of an action potential in *Aurelia*. This was further evidence that there was a "system in Coelenterates which behaves in the same way as the nerves of higher animals. The existence of an all-or-nothing response, and a refractory period is characteristic of nerve fibres." He also mentioned that he had been able to confirm Schäfer's (1878) results, but only provided details of both the histological as well as the physiological studies in later papers.

Horridge (1954a) layed "an anatomical foundation for [his] physiological studies" by repeating Schäfer's (1878) work on *Aurelia* using silver staining. His study was largely concerned with the large bipolar cells and their processes which propagate the contraction wave. The fibres he observed were of the same kind as those described by Schäfer, but he found that whereas the silver readily stained thinner fibres, the larger cells were usually not stained at all. He did not observe any preferred

orientation of the fibres, except along the edge of the bell, where few fibres ran radially, and just central to each of the tentaculocysts, where they appeared to radiate from its stalk. [It is apparent from his drawing that the latter do not belong to the giant fibre system, but to the diffuse nerve net described by Bozler (1927).] As far as synapses are concerned, Horridge noted that "it seems characteristic of coelenterate nerves that they have synapses along the axon length"; he did not see synaptic connections of the kind observed by Bozler (1927), where an axon terminated in the middle of another axon. Also contrary to Bozler, who had suggested that several systems of large bipolar cells existed in scyphozoa, Horridge found only one such system. On the smaller multipolar cells, the silver staining worked poorly, and Horridge saw no evidence that the concurrence of a large and a small axon, such as Bozler saw, was indeed the site of a synapse.

In order to record action potentials in *Aurelia*, Horridge (1954b) used a preparation that consisted of a piece of bell whose halves were connected by a bridge narrow enough that only one observable axon went across. [Although he did not give any dimensions for the width of the bridge, and it was one 'observable' axon, it is still a remarkable feat of experimental technique, given the fragility of the tissue and the neuronal density. Horridge (1954a) found that "in a field of view 2mm. in diameter there are usually three or four axons visible and sometimes as many as ten"]. From the single fibre, Horridge (1954b) recorded an action potential at each spontaneous beat of the bell. He found that a single impulse produced a contraction, that the contraction wave could be conducted in either direction across the bell, and that a single giant fibre was sufficient and necessary for conduction to occur. Action potentials of similar appearance to those described by Horridge for *Aurelia* were later recorded by Passano (1958) from giant fibres as well as from the diffuse net in *Cyanea* and *Cassiopea*.

In addition to the symmetrical swimming movement, Horridge also observed asymmetrical local movements in both the ephyra of *Aurelia*, as well as in adult

scyphozoa. In the ephyra (Horridge, 1956a), contraction of single arms, together with the coordinated movement of the manubrium, are involved in the feeding response. In the adults of *Cyanea* and *Cassiopea* (Horridge, 1956b), compensatory movements consisting of asymmetrical beats return tilted animals to a horizontal position, and slow waves of contraction (at 1/6-1/5 of the velocity of a fast contraction wave in *Cassiopea*) occur. Since Horridge (1956b) found no histological evidence for two types of muscle fibres, and he observed that the same muscle fibres that contracted during swimming also carried out the asymmetrical movements, he suggested that these two different responses were achieved by double innervation of the muscles. Innervation by the giant fibre nerve net (GFNN) led to the swimming beat, while innervation by the diffuse nerve net (DNN), which consisted of 'all of the peripheral nervous system except for the through-conducting giant fibre net of the contraction wave' resulted in local contractions. However, Anderson and Schwab (1981) found that, in *Cyanea*, the smooth muscle of the perirhopalial tissue [the area below the tentaculocyst and bound on two sides by radial muscle bands], which does not contract during swimming, contributed to the slow contractions, and suggested that it also had a part in righting behaviour. They assumed that the smooth muscle was innervated by the DNN. [There is also smooth muscle in (the ephyra of) *Aurelia*.] In the adult *Aurelia*, the ephyral feeding response does not occur due to the change in shape, and since Horridge observed neither a slow wave across the bell nor an asymmetrical component in the contraction of tilted animals, he concluded that double innervation was absent in the adult. However, according to Passano (1982), an asymmetrical response in the adult does occur when the remaining tentaculocyst is uppermost.

In *Cassiopea*, Horridge (1956b) confirmed Mayer's (1916) observation that the amplitude, but not the frequency, of the contractions in an entrapped wave preparation increased temporarily following a local stimulus. Horridge suggested that the contraction wave was maintained due to GFNN activity, but that the strength of the

contractions was influenced by activity of the DNN. He did not mention the effect of facilitation, which, according to Bullock (1943), determined the strength of the beat. [In an entrapped wave preparation, facilitation of strength of GFNN-induced muscle contraction quickly disappears (Passano, personal communication).]

Whereas his previous attempts (Horridge, 1954a) to visualize the diffuse nerve net in adult *Aurelia* had either produced no results with methylene blue, or cells that were poorly stained with silver, his methylene blue stain was successful in the ephyra of *Aurelia* (Horridge, 1956a) and in adults of *Cyanea* and *Nausithoe* (Horridge, 1956b). In all cases, the appearance of the diffuse net was similar. The cell bodies were smaller than those of the giant fibre net and frequently multipolar, and the fibres were thinner and with many bends. The fibres often appeared continuous from cell to cell, but Horridge cautioned that this should not be taken as evidence for continuity, because the contiguous giant fibres often appeared as a continuous net when over-stained. The diffuse net extended all over the epithelium, including the oral arms and tentacles, and also connected with the sensory cells, which were particularly abundant around the margin of the lappets. Horridge (1956b) showed that stimulation of the marginal lappets of *Cyanea*, where giant fibres are absent, resulted in a localized contraction followed by a swimming beat. The latter was initiated from the tentaculocyst as excitation in the diffuse net arrived there from the lappets. Direct stimulation of the giant fibres produced a "normal" contraction wave. Passano (1960), simultaneously recording the activities of the two nerve nets from the stalks of tentaculocysts, found that activity in the diffuse nerve net could cause excitation in the giant fibre net in *Cassiopea*, but, contra Horridge (1956b), did not appear to affect the ganglia in *Cyanea*.

In the ephyra of *Aurelia*, Horridge (1956a) described a diffuse nerve net which, in addition to having local sensory and motor functions, also acted on the rhythm of the "marginal ganglia" [the concentration of nerve cells in and around the stalks of the

tentaculocysts] and inhibited swimming during feeding. Horridge (1956b) could find no evidence for double innervation in the adult *Aurelia*, but provided no experimental details.

Horridge (1959) also investigated the role of the tentaculocyst in the swimming rhythm. Previous results had indicated that the rhythm originated in the tentaculocyst and could be modified by stimulation of distant regions of the bell (Romanes, 1877); and that sensory cells in and around the tentaculocyst could also influence the frequency (Bozler, 1926), suggesting that sensory information was converted into motor impulses. Horridge (1959) recorded the rhythm under various conditions in isolated segments bearing a single tentaculocyst. In undisturbed segments of *Aurelia*, he observed a high variability of the intervals between beats, and an average frequency of less than half the maximum frequency that could be achieved with stimulation. Electrical stimulation of the diffuse nerve net, which Horridge accomplished by applying stimuli to the tentacular margin of the bell outside the region of the giant fibre net, resulted in an initial acceleration of the rhythm, after which it either slowed down or was abruptly terminated by a long pause. Horridge also observed some examples where the rhythm temporarily slowed down following a mechanical stimulus at a distance from the tentaculocyst, and pointed out that a similar behaviour existed in the ephyra, which stops beating when one arm touches a food particle.

When an extra beat is forced into the rhythm by stimulating the giant fibre system, the interval following the extra beat is sometimes longer than before. Bethe (1903) had suggested that this pause compensated for the induced shorter interval. In contrast to Bethe, Horridge (1959) found that, while there was a pause when an extra beat was forced on a normal rhythm, there was no evidence that the pause was indeed compensatory, but the fact that there was a pause indicated a self-regulatory mechanism within the tentaculocyst. When a beat was forced on a rhythm that was rapid due to stimulation of the diffuse nerve net, the interval following the intercalated beat was

usually similar to the other intervals, *i.e.* that the rhythm was simply reset at the same frequency as before. Horridge deduced that, if an artificially induced beat could reset the rhythm, presumably impulses from the most rapid ganglion could also reset the rhythm of the other ganglia.

Horridge (1959) confirmed that sensory cells on and around the tentaculocysts could influence the rhythm by showing the effects of light and gravity. Increased light intensity caused an acceleration of the swimming beat in intact *Aurelia*, and preparations with a single tentaculocyst slowed their rhythm when shaded from daylight. In *Cyanea*, which has no pigment spots, differences in light intensity appeared to have no effect (Horstmann, 1934). Repeated inversions of segments, so that the subumbrellar surface (and thereby the tentaculocyst with the statocyst) was alternately facing up or down, resulted in a slower and more variable rhythm in the inverted segments. It should be noted that Horridge is not absolutely clear on the exact orientation of the inverted or "upside down", and consequently the "right way up" segments. Freely swimming animals swim upwards with the exumbrellar side up, and while they can be induced to swim downwards with the exumbrellar side down, most do not spend extended periods of time in that position (*Cassiopea andromeda*, like all *Cassiopea* species, rests subumbrellar side up on the bottom in shallow water). Therefore, "right way up" would be exumbrellar side up, but that would mean that Horridge obtained his results in the other experiments from inverted segments, since one could infer from the text (*e.g.* the mention that stimuli were applied "outside the area of the giant fibre net" is meaningless if the exumbrellar surface was facing up, since there are no giant fibres there) that the subumbrellar surface was facing up. On the other hand, if Horridge referred with "right way up" to the position of the segments in all his previous experiments, the orientation of the inverted segments would then correspond to the "normal" orientation of intact animals. In that case, his result of a slower rhythm in the inverted segments corresponds with Horstmann's (1934) finding

that the beat in *Aurelia* was highest when the subumbrellar side was up. However, in *Cyanea*, the rhythm was fastest when the exumbrellar side was up. Not only are the results different for different species and different authors (Passano, 1982), but results from isolated segments cannot necessarily be extrapolated to the whole animal. In the inversion experiment, only eleven out of twenty segments showed a significant slowing of the rhythm upon inversion, and Horridge cautioned that the frequent turning over of the segments stimulated the rhythm so that it could not even be compared to the "normal experimental rhythm", *i.e.* the rhythm from isolated segments rather than intact animals. Finally, the fact that the rhythm of the intact animal is more regular than that of a segment should be taken into consideration not just for the results of the inversion, but also the other experiments.

Whereas Horridge (1959) found no evidence that drugs such as acetylcholine, adrenaline, curare, histamine, and 5-hydroxytryptamine (5-HT) had an effect, tryptamine (10^{-5} g/ml - 10^{-4} g/ml) accelerated the rhythm in *Cyanea*, but acted slowly, and was 10-100 times less effective in *Aurelia*. In contrast, Passano (1982) reported that 5-HT (10^{-4} M) blocked the pacemaker and nerve nets in *Cassiopea*, but agreed with Horridge that "the pacemaker is pharmacologically different from analogous mechanisms in other phyla".

Horridge (1959) pointed out that the "marginal ganglia" exhibited two characteristics of "higher" nervous systems: the production of spontaneous impulses, and the integration of sensory input. In the ephyra, he (Horridge, 1956a) had shown histologically that both nerve nets extended processes into the tentaculocysts, which contained four types of neurones, and proposed the presence of a pacemaker whose frequency was modified at a polarized junction. At this junction, sensory input from the diffuse nerve net would be integrated to modulate the rhythm, but impulses would not pass in the reverse direction. Horridge (1959) interpreted his physiological data in terms of one integrating junction and one pacemaker in each ganglion, but, given the

large number of neurones in the tentaculocyst, suggested a reduplication of pacemakers, with the fastest setting the pace.

In their electron microscopical study of a tentaculocyst, Horridge and Mackay (1962) pictured a one-way synapse, but did not commit themselves as to its possible connection with the junction mentioned above. They did, however, mention that it was likely that physiologically polarized synapses would be morphologically asymmetrical. They also showed several symmetrical [Bullock's (1940) 'unpolarized'] synapses, which they thought were between axons from one nerve net, "or at least one system of fibres" [they must have entertained the idea that there were subsets of neurones]. The synapses were likely to be between axons of sensory cells, since they were the most abundant in the tentaculocyst. The nervous tissue exhibited several characteristics typical of nerve cells of other animals, such as the presence of mitochondria, Golgi bodies, microtubules, and several types of vesicles, including synaptic vesicles. There were no glial cells, which suggested to Horridge and Mackay (1962) that the neurones supplied their nutritive requirements themselves. In their conclusion, Horridge and Mackay (1962) pointed out that "the diversity of nervous systems in coelenterates lies in the arrangement of the neurones in various patterns of nets and ganglia, while the cytological details of the cells themselves is rather constant". It was therefore likely that the nervous structures they described were typical for the phylum, and could also be helpful in the identification of neurones in the peripheral nervous system.

Passano (1965) shed further light on pacemaker activity by recording from the tentaculocysts in *Cassiopea* and *Cyanea*. He had previously (Passano, 1958) recorded action potentials from the subumbrellar surface that were similar to those shown by Horridge (1954b). When recording from the tentaculocysts, he found that the wave forms of impulses from the giant fibres were consistently and distinctly different depending on whether the impulses were outgoing, *i.e.* originating from the

pacemaker, or incoming, *i.e.* originating elsewhere. A giant fibre impulse from one marginal ganglion that initiates a contraction affects all the other ganglia, and Horridge (1959) had deduced from his results that the fastest ganglion could presumably reset all the others. Passano (1965) studied the electrical activity in a *Cassiopea* ganglion following incoming impulses from the giant fibre system. The arriving impulse could either initiate a second impulse following a brief delay, or caused no local activity, presumably resetting the pacemaker without triggering an impulse. The swimming beat was not affected by the induced second impulse because of the refractory periods of the giant fibre system and the subumbrellar muscles. In *Cyanea*, the shape difference between incoming and outgoing impulses was also evident, but the response delays after incoming impulses was much longer than in *Cassiopea* (295ms versus 50ms).

Passano (1965) also investigated the effects of impulses from the diffuse nerve net on the marginal ganglia. When a giant fibre impulse was initiated by a pacemaker following an incoming impulse from the DNN, Romanes (1877) had shown that the "period of latent stimulation", *i.e.* the interval between the arrival of the DNN stimulus and the response of the pacemaker, was at least 0.5s long. Passano showed an example from *Cassiopea* where the temporal delay between the two impulses was 0.89s. However, when he monitored the excitation state of a pacemaker from *Cassiopea* for several hours, Passano recorded a range of temporal delays, and showed that the previous activity of the pacemaker had an effect on the length of the interval. If the pacemaker had just been reset by GFNN activity prior to the arrival of the DNN impulse, delay times were long, but if the DNN impulse arrived at least 1.5s after the previous GFNN impulse, the refractory state of the pacemaker had disappeared and delays were shorter. The shortest delay (0.68s) occurred in response to two DNN impulses, when the first (non-effectual) impulse had facilitated the pacemaker's responsiveness to the second one. The facilitation lasted for several seconds, and Passano suggested that it continued even after the pacemaker had initiated a GFNN

impulse. Passano also pointed out that he observed the greatest amount of spontaneous pacemaker activity when the interval between two GFNN impulses was 4-10s, which corresponded to the normal interval between contractions in undisturbed animals.

The DNN impulses Passano recorded were similar to the compound potentials of the giant fibres but were conducted at about one third the speed (14cm/s), and never originated at a "marginal center" [according to Passano (1982), input from the sensory cells associated with the tentaculocysts, which, as shown by Horridge (1959) can influence the rhythm, is direct, *i.e.* without involvement of the DNN]. The DNN impulses were not directly induced by GFNN activity, and were conducted everywhere on the subumbrella, including the manubrium, where they could cause contractions after some delay regardless of their effect on the marginal ganglia. Passano elicited several trains of DNN impulses which were equally effective in causing a contraction of the manubrium after a delay of about 2min, but which, depending on their frequency, caused the initiation of either several, one, or no GFNN impulses in the marginal pacemakers (the more rapid the stimuli, the higher the activity).

In *Mastigias*, Passano (1965) observed that the subumbrellar muscles responded with a weak contraction to a DNN impulse and with a strong contraction to a GFNN impulse. In both cases, the size of a subsequent response to either impulse was facilitated. In contrast to Horridge (1956b), Passano did not find any direct effect of the DNN on subumbrellar muscles in *Cassiopea*. [He did not deny the presence of slow or asymmetrical contractions, but thought that they were more likely explained by mechanisms other than double innervation, *e.g.* by the existence of additional conduction pathways (Passano, 1982)]. The conduction velocity of some of the slow contractions (0.15-2cm/s) in *Cyanea*, for example, disagrees with a conduction by the DNN, although the difference is less pronounced in *Cassiopea* with 8cm/s for the slow contractions and 14cm/s for the DNN. Passano also pointed out that the conduction was probably too slow for any postulated epithelial conduction as well [for which there

is no evidence in the scyphozoa.] Passano (1965) did find that a single DNN impulse was able to increase the response of the subumbrellar muscles in *Cassiopea*. In this case of 'heterofacilitation' (as opposed to facilitation by a GFNN impulse), not only was the amplitude of the GFNN-induced contraction increased, but also the neuro-muscular delay was reduced up to 20% (Passano, 1982).

While the understanding of both the diffuse nerve net as well as the giant fibre net was increasing, information regarding the endodermal nerve net was [still is!] sparse. Bozler (1927) had briefly mentioned it in *Rhizostoma*, and Horridge (1956a) described neurones extending towards the stomach from the inside of the manubrium in the ephyra of *Aurelia*. Passano and Passano (1971) carried out the first in-depth study of the endodermal nerve net in *Phacellophora* and *Cyanea*. Staining with methylene blue revealed a synaptic net consisting of bipolar and a few tripolar cells. The processes, which were unbranched, ended on other processes. Cell size was larger in larger animals, but the density of the net decreased. Passano and Passano likened the appearance of the endodermal net to that of the DNN, but did not find clear evidence for primary sense cells.

Anderson and Schwab (1981) mentioned that neurons "are quite common" in the endoderm of *Cyanea*. At the EM-level, they occasionally found interneural synapses, but did not identify any neuroeffector synapses. Both ecto- and endodermal neurons had many features typical of neurons, but many also contained an unusual, large vacuolar-like structure with unknown function. Interneural synapses were similar to the ones described by Horridge and Mackay (1962). In synapses between GFNN neurons, synaptic vesicles were symmetrically distributed, with a single layer of 6-7 vesicles against each membrane. Anderson and Schwab did not find gap junctions, and intracellular injection with Lucifer Yellow did not show dye coupling. [Gap junctions have not been found in the Scyphozoa, but are present in the Hydrozoa (Spencer, 1989), and although 'typical' gap junction plaques have not been found in the

Anthozoa, intercellular coupling appears to be present in *Renilla* (Germain and Anctil, 1996)]. Neuromuscular and interneural synapses were very similar in terms of number and size of vesicles, but the former were not symmetrical. In their three-dimensional diagram of the ectodermal tissue, Anderson and Schwab showed the location of the GFNN neurons amongst the muscle tails at the bases of the myoepithelial cells.

In order to gain direct access to the neurons, Anderson and Schwab (1984) removed the epithelial cells by exposing the tissue to either osmotic shock, using 20% SW, or oxidation, using a diluted bleach solution. An epithelial cell-free preparation allowed Anderson (1985) to examine the mode of operation of the interneural synapses further. He recorded intracellularly from synaptically connected cells, and found that conduction occurred equally well in either direction with a constant synaptic delay, which confirmed that the morphologically symmetrical synapses were indeed physiologically nonpolarized (bi-directional), chemical synapses. Morphological reconstruction of the synapse (Anderson and Grünert, 1988) revealed its three-dimensional organization and confirmed previous results (Anderson and Schwab, 1981), *e.g.* regarding the small number of synaptic vesicles. Whereas the neurotransmitter used by neurons of the GFNN at these synapses has not been identified yet, Anderson *et al.* (1992) have shown the presence of RFamide-like material in the DNN of scyphomedusae. In fact, peptides resembling the molluscan neuropeptide FMRFamide are ubiquitous throughout the phylum, and a whole family of RFamide-like peptides has been identified (Grimmelikhuijzen *et al.*, 1989).

Details of synapse physiology and ionic-electrical currents in scyphomedusae are covered by Spencer (1989) and Anderson (1989), respectively. As the present thesis is primarily concerned with structural and developmental neurobiology, no attempt will be made to review the modern physiological work in detail. Spencer (1989) pointed out that it was possible to interpret earlier experiments such as the ones by Bullock (1943), which revealed the effect of excess magnesium on conduction and muscle

contraction, as indicating that both the neuro-neuronal and the neuromuscular synapses required calcium for transmitter release. Bullock (1943) had found that excess magnesium depressed facilitation both at the neuromuscular and neuro-neuronal synapses, and Bethe (1908) had already reported its "paralyzing" effect on medusae. According to Spencer, it has "become apparent that many of the basic synaptic mechanisms and properties that we associate with more "advanced" nervous systems can be demonstrated in the Cnidaria." Similarly, with regards to ionic currents, Anderson (1989) stated that "the variety of ionic currents present in the scyphozoans closely resemble those found in higher animals." Anderson had found evidence for sodium, calcium, potassium, and chloride currents in *Cyanea*.

Thus, with the evidence available so far, it appears that Bullock and Horridge (1965) were justified in stating that "the structural and functional elements of the nervous system of coelenterates are not fundamentally different from those in higher animals."

Objectives

Despite the extensive research outlined above, many unanswered questions remain, *e.g.* what is the nature of the neurotransmitter used by GFNN neurons? The GFNN and the DNN have been documented, but are there additional pathways as proposed by Horridge (1959) and Passano (1982)? As to the concept of double innervation of the striated muscles, can it alone explain some behaviours, as suggested by Horridge (1956b), or should it be amended (Anderson and Schwab, 1981), or be regarded as only one (unlikely) of several possible mechanisms (Passano, 1982)? The similarities between the coelenterate nervous system and that of 'higher' animals have been pointed out, but there are still a number of substances, such as thyroxine and

retinoic acid, with a known effect in 'higher' systems whose effects in coelenterates have not been tested at all or only to a limited degree.

For the hydromedusae, Passano (1965) suggested that "it will be instructive to attempt to follow the various classes of units backward in development through the metamorphosis out of which they have emerged from the polyp; possibly some of the seeming complexity of these medusa is due to their successive developmental stages each requiring certain control systems, so that what we are able to record is a functional collage of superimposed elements reflecting ontogenetic as well as phylogenetic succession." [Haeckel would have been thrilled, as I am]. The Scyphozoa are less complex than the Hydrozoa; nevertheless, Passano's suggestion is just as applicable to them. Scyphozoan medusae are more complex than the polyps, possibly reflecting ontogenetic succession of elements. A study on scyphozoan development, however, would approach the question of phylogenetic succession from a less complex system; perhaps 'succession' should be replaced by 'precession'. In fact, if Werner (1975) is right and the Scyphozoa are the stem group of the phylum, what we learn from them might more closely resemble the ancestral conditions.

I have attempted to take Passano's suggestion one step further by not stopping at the polyp stage, but by examining the planula as well, and thus the whole life cycle. Given their evolutionary significance, *e.g.* for possessing the supposedly most 'primitive' nervous system, the Scyphozoans along with the other coelenterates have been studied from many points of view; yet, despite their significance, at the time of writing there are no attempts to follow any scyphozoan species through from planula to adult from the point of view of developmental neurobiology. Furthermore, published accounts of the neural architecture of individual life cycle stages are sometimes few in number and with contradicting results (as for the scyphistoma), or deal with only some aspects of neuroanatomy (such as the tentaculocyst of the ephyra). Of the studies that do exist, many use methods that are now considered conventional, such as electron

microscopy or methylene blue staining. The failure to identify neuronal subsets by conventional histological means clearly indicates the need for more selective methods.

The fate of the nervous system in animals that develop by metamorphosis through a series of life stages is of special interest and has been thoroughly studied in holometabolous insects. A scheme for the hormonal control of insect metamorphosis has been elucidated, and the restructuring of the nervous system has been studied. New sets of neurons are generated, while some nerves die or take over new functions (Truman *et al.*, 1985). Although strictly speaking metamorphosis in *Aurelia* cannot be compared to that in insects, since in the latter, an entire life stage metamorphoses to the next stage, while, at least as far as the development of the ephyra from the strobila is concerned, the scyphistoma continues to exist after the release of the ephyrae, the fate of the neurons is nevertheless of interest. If new neurons are added, where do they come from? The Hydrozoa possess interstitial cells, which are also referred to as replacement cells, since they are able to replace certain cell types. The Scyphozoa, however, do not possess interstitial cells. Instead, they have amoebocytes which are weakly phagocytic (Chapman, 1974), and while they have the appearance of undifferentiated cells, it is not clear whether they can replace neurons or not.

Therefore, the objectives of this study are as follows:

- to document the structure and distribution of the nervous system of *Aurelia aurita* at each stage of its development, and, in an attempt to expand on the results of previous studies (when available), to do so by using both conventional and modern methods.
- to determine whether subsets of neurons can be distinguished by using more specific methods and techniques, particularly in regard to labels and dyes. As far as the labels are concerned, I have generated neuron-specific monoclonal antibodies, which has not been accomplished before in scyphozoans, and as far as the dyes are concerned, I have monitored the activity-dependent uptake of the dye rhodamine B, which has not been done before in coelenterates in general.

- to determine the extent of the involvement of the nervous system in developmental processes. I have manipulated specimens of different life cycle stages by subjecting them to various drug treatments, such as thyroxine, retinoic acid, and FMRamide, which are known to influence elements of the nervous system.

Lastly, in order to determine how *Aurelia* compares to other scyphozoans, I have carried out some of the experiments on other scyphozoan species that were either locally available or were contributed by researchers from various locations.

2. GENERAL MATERIALS AND METHODS

The materials and methods described in this section are those that apply to several of the following chapters. Modifications or individual materials and methods are provided in the appropriate chapters when indicated.

Animal Maintenance

Japanese and Californian *Aurelia aurita*, *Pelagia colorata*, *Chrysaora fuscescens*, and *Cassiopea* sp. were kindly provided by Freya Sommer from the Monterey Bay Aquarium, Monterey Bay, California, USA. Locally, *Aurelia aurita*, *Phacellophora camtschatica*, and *Cyanea capillata* were collected off the docks of Brentwood Bay, Pedder Bay, and Oak Bay, B.C., and of Friday Harbor, WA, USA. *Cassiopea xamachana* were also collected by Daryl Parkyn and Debra Murie from the waters of Key West, FL, USA. All animals were kept in the seawater system in the Biology Department, University of Victoria, at 11°C ($\pm 4^\circ\text{C}$). The animals were fed *Artemia nauplii* daily.

Electron Microscopy

Transmission Electron Microscopy

Specimens were fixed for 2 h at 4°C or on ice in 2.5% glutaraldehyde, or 2.5% glutaraldehyde and 1% acrolein, in 0.01M- 0.4M cacodylate buffer, pH 7.2. They were then washed in the same buffer, postfixed for 1 h in buffered 1% OsO₄, and again thoroughly washed in buffer. Specimens were then enbloc stained overnight at 60°C in 2% aqueous uranyl acetate and washed in distilled water. After dehydration in a graded ethanol series, they were embedded in Epon, using propylene oxide as the transitional fluid. Blocks were sectioned on a Reichert UM2 ultramicrotome and sections viewed on a Philips 300 or a Hitachi 7000 electron microscope.

Scanning Electron Microscopy

Specimens that had been fixed and dehydrated as for TEM were critical point-dried using liquid CO₂ in a Bomar SBC-1500 critical point dryer, mounted on SEM stubs, gold-coated in an Edwards S150B sputter coater, and viewed on a JEOL JSM-35 scanning electron microscope.

Monoclonal Antibodies

Production of Monoclonal Antibodies

Monoclonal antibodies were produced using a modification of the technique pioneered by Köhler and Milstein (1976). Ephyrae were exposed to 50% sea water and

dissociated by forcing several animals at a time through 18 to 26 gauge needles. The suspension was centrifuged at 1800 g and the cells were resuspended in sterile PBS (0.14 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M K₂HPO₄), pH 7.4. A female BALB/c mouse (Charles River, Canada) was injected intraperitoneally (IP) with approximately 10⁶ cells in 0.25 ml of PBS plus complete Freund's adjuvant (1:1). Three weeks after the first injection, the mouse received an IP booster injection with the same inoculum, except that Freund's incomplete adjuvant was used. Three days after boosting, the spleen cells of the mouse were fused with SP2/0 myeloma cells and cloned in methylcellulose (Terry Fox Laboratories, Vancouver, B.C.) (Davis *et al.*, 1982). After clones had grown to approximately 0.5-1 mm in diameter, they were removed using sterile pipet tips, and resuspended in wells of 96 well tissue culture plates containing 200µl of RPMI 1640 medium containing 1% hypoxanthine/thymidine, 20% bovine calf serum and 1 * 10⁶/ml BALB/c thymocytes. When the pH had decreased in the wells as indicated by a colour change of the supernatant to yellow, the supernatants were screened for antibody activity by immunofluorescence (see below).

Positive clones were recloned twice by limiting dilution to ensure monoclonality (Goding, 1986), retested and expanded in 24 well tissue culture plates and tissue culture flasks.

Ascites fluid was generated in mice primed with 0.5 ml pristane injected IP, followed two weeks later by an IP injection of 10⁶ hybridoma cells. Ascites was harvested from swollen mice either by draining the peritoneal cavity of live mice using an 18 gauge needle, or by opening the abdominal cavity of sacrificed mice and removing the fluid and cells aseptically with a Pasteur pipette. After centrifugation (20 min., 900g), the fluid was stored at -20°C. When the cells of the ascites fluid were to be saved, 2.5ml of RPMI 1640 were injected intraperitoneally and the acites fluid was drawn out. It was centrifuged (5 min., 700g) and the supernatant tested. Cells still producing suitable antibodies were grown up further in tissue culture, or frozen in

liquid nitrogen. For the latter, 5×10^5 cells were resuspended in one ml of 90% fetal bovine serum with 10% dimethyl sulfoxide, and stored in cryotubes (Corning). The tubes were sealed and frozen on dry ice for 1h, after which they were stored in liquid nitrogen at -187°C .

Isotyping

Antibodies from doubly cloned hybridomas were isotyped by a sandwich ELISA (enzyme-linked immunosorbent assay) with a kit using class and subclass specific anti-mouse antibodies (anti IgM, anti-IgA, anti-IgG₁, anti-IgG_{2a}, anti-IgG_{2b}, and anti-IgG₃) (American Qualex, La Mirada, CA) according to the instructions supplied by the manufacturer.

Determination of Apparent Molecular Weight of Antigen

Polyacrylamide gel electrophoresis and immunoblots were performed according to Beecroft *et al.* (1993). Ephyrae were dissociated as for the production of monoclonal antibodies, except that the cells were resuspended in Laemmli buffer (Laemmli, 1970). The solution was boiled for 3 min. and then centrifuged for 30s at 14,000g. The optimal protein loading was determined by separating the proteins from the titrated supernatant on a 12.5% Laemmli gel under reducing conditions, followed by staining the molecules with Coomassie blue R-250. For the immunoblot, 15- 20 μl of 0.05-1.3 $\mu\text{g}/\mu\text{l}$ protein were separated in the same way and transferred onto polyvinylidene difluoride membranes. Bound antigens were detected with an Immun-Blot assay (Biorad, Richmond, CA) using alkaline phosphatase conjugated second antibody with BCIP/NBT (Bromochloroindolyl phosphate/Nitro blue tetrazolium) colour development reagents.

Immunohistochemistry

Immunofluorescence

Specimens were fixed at 4 °C in Zamboni's (1967) fixative for 2 h and then washed several times in PBS (0.14 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M K₂HPO₄), pH 7.2. The monoclonal antibodies were either used as neat supernatant to which 0.01% Triton X-100 and 0.03% sodium azide were added, or as ascites fluid, diluted 1:10⁵ to 1:10⁶. Antibodies were diluted in Tris buffer (0.05 M Tris Base, 0.17 M NaCl, 0.06 M Na₂S₂O₃), pH 7.0, plus 0.01% Triton X-100 plus 0.03% sodium azide. Both primary and secondary polyclonal antibodies were used at a dilution of 1:100. Primary polyclonal antibodies used were rabbit anti-FMRFamide (Immunoclear Corporation, Minnesota, USA). Secondary antibodies used were goat anti-mouse IgG+M (Fab) and goat anti-rabbit IgG (H+L), and were labelled with either FITC or rhodamine (Jackson ImmunoResearch Laboratories, Inc., Mississauga, ON).

All incubations were carried out at room temperature with agitation on a rotator table. Specimens were incubated overnight in the first antibody, washed several times in Tris buffer (typically 3*15 min.), and incubated for 2 h to overnight in the secondary antibody. After several washes in Tris buffer, the specimens were mounted in PBS-glycerol (1:1) to which 0.03% sodium azide, and 0.3% N-propyl-gallate had been added, and viewed with a Leitz Fluorescence microscope. For the controls, the first antibody was either omitted from the incubating medium, or antibodies non-relevant for the preparation (NRA) were used (CA7AE, a monoclonal IgM antibody recognizing *Leishmania donovani* lipophosphoglycan, donated by T.W. Pearson, Biochemistry Department, UVIC, and rabbit anti-GnRH, donated by N. Sherwood, Biology Department, UVIC).

Immunogold labelling

The procedure followed was based on the method by Singla and Mackie (1991). When anti-FMRamide was used as the first antibody, specimens were fixed for 2 h at 4°C in a solution of 0.1% glutaraldehyde and 4% paraformaldehyde in 0.3M cacodylate buffer, pH 7.2. When mAb Aa was used as the first antibody, the glutaraldehyde was omitted from the fixation solution and only 2% or 4% paraformaldehyde was used. Specimens were then rinsed in the same buffer and osmicated in 1% buffered osmium tetroxide for 1 h at 4°C, followed by a brief rinse in distilled water. The specimens were placed directly into absolute ethanol followed by propylene oxide and were then embedded in Epon-Araldite mixture. Thin sections were mounted on Formvar-coated 100 mesh nickel grids. The grids were placed on a drop (50µl) of distilled water for 10 min., followed by 30 min. on a saturated solution of sodium metaperiodate, 2*5 min. on distilled water, and 15-30 min. on normal goat whole serum diluted 1:20 with PBS-Tween-BSA (0.01M sodium phosphate buffer, pH 7.2, 0.5M NaCl, 0.5% BSA and 0.05% Tween 20 with 0.03% sodium azide). They were then incubated for 1 h on Ramide antiserum diluted 1:1000 with PBS-Tween-BSA or on mAb Aa supernatant diluted 1:10, washed for 3*5 min. on the same buffer, and then incubated for 1 h on 5nm or 15 nm gold-conjugated goat anti-rabbit IgG or goat anti-mouse IgG+M (Jackson ImmunoResearch Laboratories, Inc., Mississauga, ON) diluted 1:10 with buffer. After that, they were washed for 3*5 min in the same buffer and for 2*5 min on buffer with NaCl (0.15M NaCl, 0.01M sodium phosphate buffer, pH 7.2). After the final rinse, specimens were postfixed for 15 min on a drop of 2.5% glutaraldehyde in PBS-Tween-BSA, rinsed in distilled water, and stained for 30 min with 5% uranylacetate in 50% ethanol, after which they were rinsed in 50% ethanol, and then in distilled water. For controls, the first antibody was either omitted from the incubating solution, or anti-GnRH or mAb CA7AE were used. In addition,

anti-FMRFamide was preabsorbed overnight with 10 $\mu\text{g}/\text{ml}$ of synthetic FMRFamide prior to use.

Staining F-Actin with Fluorescent Phallotoxins

Stock solutions were prepared by dissolving 300 units of NBD phalloidin or rhodamine phalloidin (Molecular Probes, Eugene, OR) in 1.5 mL of methanol, resulting in approximately 6.6 μM solutions. Prior to use, 5 μl of either solution were dried and then redissolved in 200-1000 μL of Tris buffer or PBS. Specimens were stained in this solution for 3-5 min at room temperature, washed twice rapidly in buffer, and mounted on a slide in a 1:1 solution of PBS and glycerol, to which 0.03% sodium azide and 0.3% N-propyl gallate had been added.

3. EXPERIMENTAL STUDIES

The Planula

Introduction

The term 'planula' was introduced in 1877 by Lankester (Hyman, 1940) to denote the diploblastic metazoan ancestor, which he regarded as a solid rather than a hollow organism. Metschnikoff (1882) also favored this view and called the former "parenchymula". They disagreed on this point with Haeckel (1874), who postulated in his 'Gastraea-Theory' that the common ancestor of all metazoa is a two-layered organism, the 'Gastraea', which is reproduced in metazoan embryology as the gastrula,

and whose layers are retained in all metazoa as ectoderm and endoderm. While all three agreed that the metazoa were derived from colonial flagellates, represented by a hollow ball consisting of a single layer of cells (Haeckel's 'Blastaea'), one of the points on which their views differed was the manner of endoderm formation. According to Haeckel, the latter was achieved by invagination, to Metschnikoff, by multipolar ingression, and to Lankester, by primary delamination. One might assume that studies on coelenterates, which are at the beginning of the eumetazoan phylogenetic ladder and whose importance in phylogenetic research was pointed out by Haeckel (1884), could shed some more light on this point. However, the controversy continued and different modes of endoderm formation were described even for the same species. In the case of *Aurelia aurita*, Haeckel (1881) observed that the endoderm was formed by invagination and that the original mouth opening was retained in the free-swimming larva, then closed, and opened again in the same place after settlement. However, he also reported to have seen incomplete invagination in cases with direct development. Claus (1883) confirmed that a "process quite close to an invagination" occurred, while Goette (1887) insisted that in *Aurelia*, "gastrulation occurs through cell-inwandering", and Hein (1900) found that some cells indeed migrated inward in a multipolar fashion, but that those cells degenerated and did not participate in endoderm formation. Rather, the latter was achieved by an invagination with rapid division of the invaginated cells, and the blastopore persisted as a very fine canal. Hargitt and Hargitt (1910) acknowledged that variations occurred in the development of *Aurelia*, but pointed out that the differences were only of minor importance, since the end result was the same, *i.e.* the typical planula. They speculated that the different methods to attain this result might only be indicative of variations in egg size or environmental factors. They also expressed some criticism towards some researchers (perhaps Haeckel in particular) by stating that "the controversies over these unessential points only tend to show that attempts to bring these and other processes into exact and constant agreement with

some assumed "law" are futile, and often only indications of ignorance or bias". Berrill (1949) untangled the situation somewhat when he found that, in Scyphozoa in general, the mode of gastrulation indeed appeared to be correlated to variability in egg size, and that, in *Aurelia* in particular, egg size is most variable. He pointed out that "in general, the smaller eggs gastrulate by unipolar ingression, the largest by invagination alone, while those of intermediate size combine invagination and ingression in various degrees".

In the Scyphozoa, the "end result", as the Hargitts (1910) put it, of the different processes of development, is the free-swimming and, with the exception of the lucernariid *Haliclystus* (Lesh-Laurie and Suchy, 1991), uniformly ciliated planula. Variations have been reported regarding the age at which the young leave the ovary. According to Siebold (1839), the eggs leave the ovary and reach the oral arms without the help of cilia. On the other hand, Agassiz (1862) insisted that, while there is some variation in size, the young of *Aurelia* are ciliated before they leave the ovary. After their release from the oral arms, the ovoid planulae swim with their broadened anterior end pointed forwards.

Attachment takes place at the anterior pole, which in some species develops an indentation just prior to settlement, while the posterior pole develops into the oral region of the future polyp. 'Attachment' is here referred to as behaviour which can occur repeatedly, whereas 'settlement' is generally permanent and generally occurs prior to metamorphosis. Settlement is not random, but rather, scyphozoan planulae preferentially attach to the underside of horizontal surfaces (Brewer, 1978). Some, *e.g.* *Cyanea*, also show a marked preference for rough rather than smooth surfaces (Brewer, 1976), while others, *e.g.* *Aurelia* (Brewer, 1978) and *Chrysaora* (Cargo, 1979) are less discriminatory regarding surface texture in experimental set-ups.

After settlement, the larvae commonly metamorphose into scyphistomae within a few days. In *Cassiopea andromeda* (Fitt *et al.*, 1987), metamorphosis does not take

place in sterile artificial seawater devoid of any organic substances, but occurs in sterile natural seawater from the Red Sea, and can be induced by cholera toxin and thyrotropine in artificial seawater. Also, older planulae metamorphose sooner and respond to lower concentrations of the exogenous inducers, which, in nature, could contribute to enhanced metamorphosis and be of advantage for the survival of the species. As for the effect of the chemicals, Fitt *et al.* (1987) suggested that, as in mammalian systems, the mechanism of action of cholera toxin could involve cyclic adenosine monophosphate (cAMP) as a messenger. However, dibutyryc cAMP, which can pass through membranes and functions normally inside the cell, does not induce metamorphosis, and levels of intracellular cAMP increase only slowly when metamorphosis is induced, suggesting that cAMP is associated with metabolic processes during metamorphosis rather than with a triggering mechanism.

Widersten (1968) examined several scyphozoan species from the west coast of Sweden and reported that they vary in size from $125 \times 75 \mu\text{m}$ to $360 \times 165 \mu\text{m}$. Nematocysts are found in the ectoderm and are species-specific in their complement, size, and distribution. In young *Aurelia aurita* planulae, atrichs and eurytelous heterotrichs are abundant, whereas older larvae also carry a few of a second type of atrich, which is (as are the other two types) also found in the polyp, and which was termed 'atrichous polyspira' by Spangenberg (1964). Unlike Haeckel (1881), Widersten did not find any budding of daughter planulae, nor any planulae that developed directly into ephyrae.

Reports on the presence of a nervous system in scyphozoan planulae differ. Widersten (1968) described nervous structures in a number of different scyphozoan species; in methylene blue stained *Aurelia* planulae, he observed a "neurofibrillous layer" consisting of only a few cells; unfortunately, he only provided schematic drawings, but no photographic evidence. In contrast to Widersten's findings, ultrastructural studies by Martin and Chia (1982) did not reveal any nervous structures

in either *Cassiopea xamachana* or *Halichystus salpinx*, both of which are composed of 4 cell types only. *Cassiopea*'s ectoderm consists of supportive cells and nematoblasts, and its endoderm of supportive and "interstitial" cells only. The ectoderm of *Halichystus* consists of supportive cells, nematocytes, and "interstitial" cells, and its endoderm of one type of supportive cell. *Halichystus* is unusual in that the core of the planula is made up consistently of only 16 linearly arranged cells, and in that the ectoderm is not ciliated. As the larva moves by an extension-retraction phenomenon involving antagonistically positioned microfilaments of the endoderm and ectoderm, an amorphous sheath covering the surface is left behind (Lesh-Laurie and Suchy, 1991).

I have examined the planulae of *Aurelia* both at the light and electron microscopic level, and attempted to obtain further evidence for the presence of a nervous system using immunofluorescence with antibodies to FMRFamide.

I have also investigated the potential involvement of the nervous system in settlement and metamorphosis by exposing planulae to FMRFamide, $MgCl_2$, retinoic acid, thyroxine (T_4), and iodine (I_2). FMRFamide-positive neurons are present in the planula, and although they presumably only comprise a subset of neurones, if the nervous system is involved in metamorphosis, it is conceivable that FMRFamide may play a role. $MgCl_2$ decreases synaptic transmission and is an effective anaesthetic for the scyphistoma and the jellyfish. Thyroxine (tetraiodothyronine) and iodine have been shown to be involved in the induction of strobilation in *Aurelia* (Spangenberg, 1967, 1971). In vertebrates, where the synthesis of thyroxine is under nervous control (Etkin, 1968), thyroid hormones bring about a number of metamorphic changes, including the differentiation of neurons which results in the formation of new neuronal pathways (Hoskins and Grobstein, 1985). While thyroxine is a 'vertebrate' hormone, its action in the scyphistoma suggests that it may also play a role in the metamorphosis of the planula.

The effect of retinoic acid, a derivative of vitamin A, has also mostly been studied in vertebrates, where it has a variety of developmental effects, including teratogenesis and pattern duplication (Byrant and Gardiner, 1992) and differentiation of neurons in the central nervous system (Muto *et al.*, 1991), but it also exhibits teratogenic and metamorphosis-inhibiting activity in the sea squirt *Ciona* (Denucé, 1991).

Since elements of the nervous system appear to be concentrated in the anterior part of the planula, I have attempted to determine the rate of metamorphosis in animals that had been cut into posterior and anterior halves. Given the experimental difficulties in obtaining such animals, the experimental set-up included exposure to filtered seawater and thyroxine, but none of the other treatments previously used. Thyroxine was chosen because of its powerful effect on metamorphosis in intact animals.

Materials and Methods

The following Materials and Methods are those not covered in Chapter 2 (General Materials and Methods).

Scanning Electron Microscopy

When possible, larvae were collected from one site on an oral arm of the same adult jellyfish, as perhaps position on the oral arm could be related to age of the planulae (*i.e.* a position closer to the disc could indicate less traveling time from the ovary, while planulae further down on the oral arm could be closer to being released and would therefore be older). After removal, the planulae were rinsed several times in order to remove as much adhering mucus from the adult as possible. They were then processed as described in Chapter 2, except that they were not critical point dried.

Instead, after dehydration in a graded ethanol series, the specimens were transferred into hexamethyldisilazane (C. L. Singla, personal communication). After several changes of the latter, the fluid was allowed to evaporate in an open dish. After the planulae had dried, they were gold-coated and mounted onto SEM stubs.

Induction of Metamorphosis

The planulae were collected and treated as for the SEM prior to fixation. 50 planulae were distributed in each of the 20 ml beakers for the treatments and controls. The planulae remained in the treatments for the duration of the experiments unless otherwise specified. The treatments consisted of (10^{-7} and 10^{-4} M) thyroxine (T_4), (10^{-7} and 10^{-5} M) iodine (I_2), (2×10^{-8} and 10^{-7} M) FMRFamide (FMRFa), 10^{-5} M iodine plus 2×10^{-8} M FMRFamide, anti-FMRFamide antibodies (1:200) in 1% DMSO, 0.13M $MgCl_2$, and (10^{-7} - 10^{-5} M) retinoic acid (RA) in seawater that had been filtered through a $0.22\mu m$ Millipore filter. The concentration of the $MgCl_2$ corresponds to the one used for anaesthetizing scyphistomae and jellyfish. For the latter, a stock solution of 0.33M $MgCl_2$ in distilled water is prepared and diluted 1:2 with seawater prior to use. Since seawater contains 30 mM magnesium, the final concentration is 0.13M. The depletion of other ions involved was considered unimportant as animals were found to survive indefinitely in the mixture and metamorphose on return to seawater. The controls consisted of filtered seawater only, H_2O (1:100), a non-related antibody, NRA (anti-GnRH), (1:200), 1% DMSO and 0.1% ethanol in seawater. The H_2O and DMSO controls were included since the lyophilized anti-FMRFamide antibodies were reconstituted in distilled H_2O and presented to the planulae in 1% DMSO in seawater. The ethanol control was included since the retinoic acid was dissolved in absolute ethanol first before being diluted with seawater. The planulae were inspected daily for signs of attachment and of metamorphosis. The beakers were covered with petri dishes

and kept in a tank in the seawater table with constant supply of flowing seawater. To determine whether the treatments produced statistically significant results, the data were subjected to the sign test. Although nonparametric tests such as the sign test do not utilize the estimation of the mean (Zar, 1974), the standard errors of the mean were included in the graphical presentation of the data in order to show the distribution of the means.

For one experimental set-up, the planulae were cut in half with a razor blade and 10 each (20 halves) were exposed to FSW and T_4 ($10^{-7}M$).

Results

Gross Morphology and Behaviour

The planulae vary in shape from almost spherical to pear shaped or almost cylindrical (Fig. 2a), and are approximately $125\mu m * 75\mu m$ in size. The anterior pole, which is more or less enlarged, is pointed forward during swimming. Locomotion is rapid and occurs by anti-clockwise rotation around the longitudinal axis (when viewed from the oral pole and in the direction of movement) due to ciliary movements. Ciliation is uniform (Fig. 2b). Some cilia have disc-shaped tips resembling those of paddle cilia. When viewed under the light microscope, the cilia at first appear straight, but after a short period of time, the disc-shaped tips can be observed. The latter can also be seen at the SEM level in chemically fixed planulae (Fig.2.c).

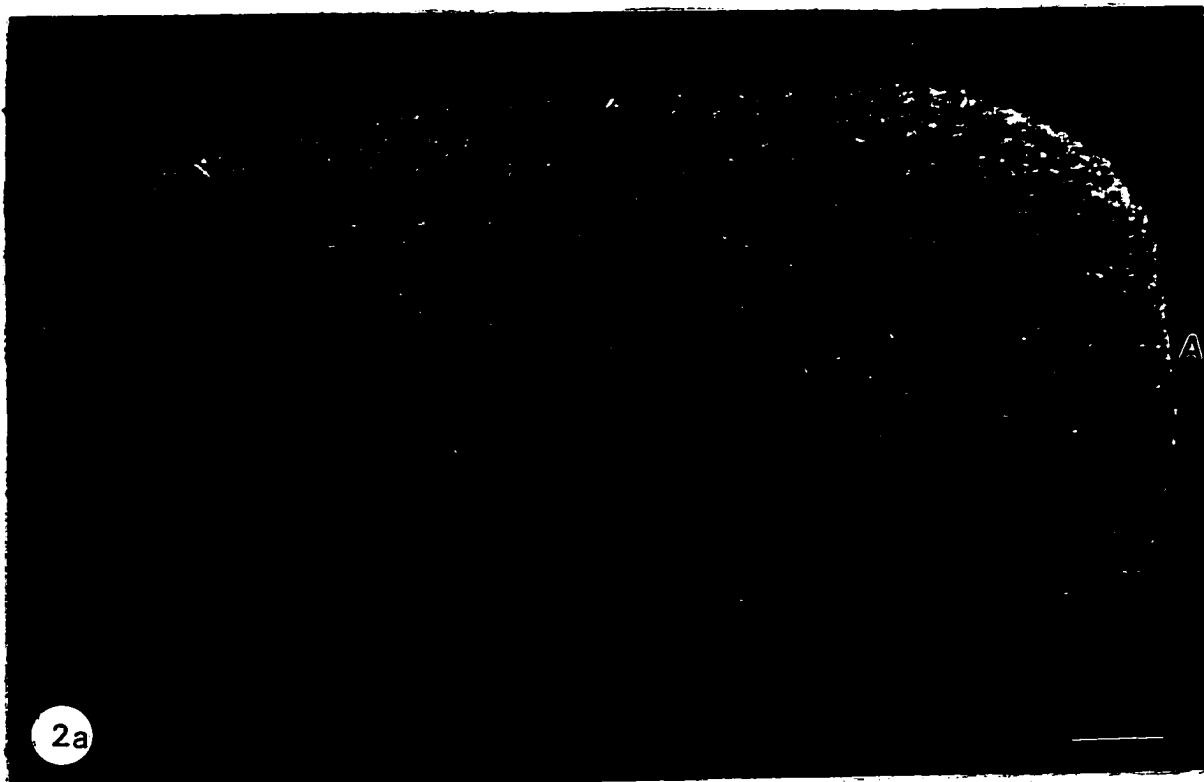
The larvae swim about freely in the dish, frequently attaching to the hyponeuston and less frequently to the bottom or sides of the dish. When attached, they continue to slowly rotate. The attachment can be brief or last for prolonged periods of time. In general, the planulae have settled before the onset of metamorphosis, but very rarely some specimens with tentacle buds were observed that

Fig. 2 Planula of *Aurelia aurita*

a) Overview, anterior end (A) thickened; SEM picture. Scale bar = 10 μ m

b) Uniform ciliation of ectodermal surface; light micrograph. Scale bar = 5 μ m

c) Cilia with collar of microvilli (arrow) and paddle cilia (arrowhead), SEM picture. Scale bar = 1 μ m



were lying on their sides. However, metamorphosis was never completed in this position; sooner or later, the animals either attached to the dish or disintegrated. Once settled, the animals did not detach and settle somewhere else, although they have the capability to do so when forced. Often, the planulae settled on the hyponeuston, hanging upside down from the water surface, and remained in this position until too heavy to be supported any longer. They also often settled in groups whose members could be at various stages of development.

The onset of metamorphosis is characterized by an indentation in the oral pole leading to the formation of the mouth and by the appearance of two tentacle buds, so that the oral region becomes enlarged and appears flattened and bilaterally symmetrical. The formation of two more tentacle buds follows, accompanied by an increase in size of the animal. At this time, the reversal of the proportions of the free-swimming planula has become more pronounced. Whereas the aboral poles are broadened in the planulae, they become narrower and more stalk-like in the developing polyps. Frequently, the latter turn cannibalistic, even though they are hardly bigger than the captured planulae. The polyps commonly develop 16 tentacles total, but variations were observed, frequently where specimens were missing one or two tentacles. In the latter case, one or two tentacles could be bifurcated. The polyps remained white until fed on *Artemia* nauplii, after which they started to turn orange-pink.

Light Microscopical Observations

Although Widersten (1968) reported having been able to visualize nervous elements in the planula of *Aurelia aurita* using methylene blue, I did not find that the stain produced reliable enough results. Antibodies to FMRFamide, however, labelled both sensory (Fig. 3a) and mostly bipolar nerve cells in the ectoderm of the larva (Fig. 3b). The columnar-shaped cell bodies of the sensory cells are interspersed among the

supportive cells. At the distal pole, each sensory cell possesses a cilium which extends into the external environment. The neuronal cell bodies as well their fibres and those of the sensory cells are located at the bases of the ectodermal cells. Due to a high background, the labelled fibres were often not easily seen. The background was more intense in younger planulae, in which also only a few cells were labelled, and mostly cell bodies could be observed. In older planulae, more nerve cells appear to be labelled in the anterior half of the animal (Fig. 3b,c). Labelled fibres are oriented mostly longitudinally along the anterior-posterior axis, particularly in the midriff section of the animal, but some fibres are oriented transversely (Fig. 3c). The fibres, particularly in the younger stages, do not seem to contact other labelled fibres, and the 'typical' appearance of a nerve net of FMRFamide-positive cells cannot be observed.

Electron Microscopical Observations

Ectodermal and endodermal cells of the planulae are separated by a thin mesogloea. The ectoderm contains supportive cells, nematocysts and -blasts, sensory cells, and ganglion cells. A cross section at the level of the ectodermal cells shows that they appear almost square and form straight borders with their neighbours (Fig. 4a). Profiles of cilia, which are of the 9+2 microtubular arrangement, and their collars of microvilli can be seen all over the ectodermal surface (Fig. 4a). The supportive cells contain a variety of inclusions (Fig. 4a,b) and membrane arrangements (Fig. 4c). Towards the apical pole of the cells, accumulations of spherical inclusions of different sizes are found. Some of the larger inclusions appear to contain a mucus-like substance and exhibit a range of electron densities, while others are more uniformly electron-lucent. A few cells contain smaller, electron-dense granules and dense-cored vesicles. The dense-cored vesicles are of different sizes and density and vary in the extent to which they are filled (Fig. 4b, d). The nuclei possess prominent nucleoli and are

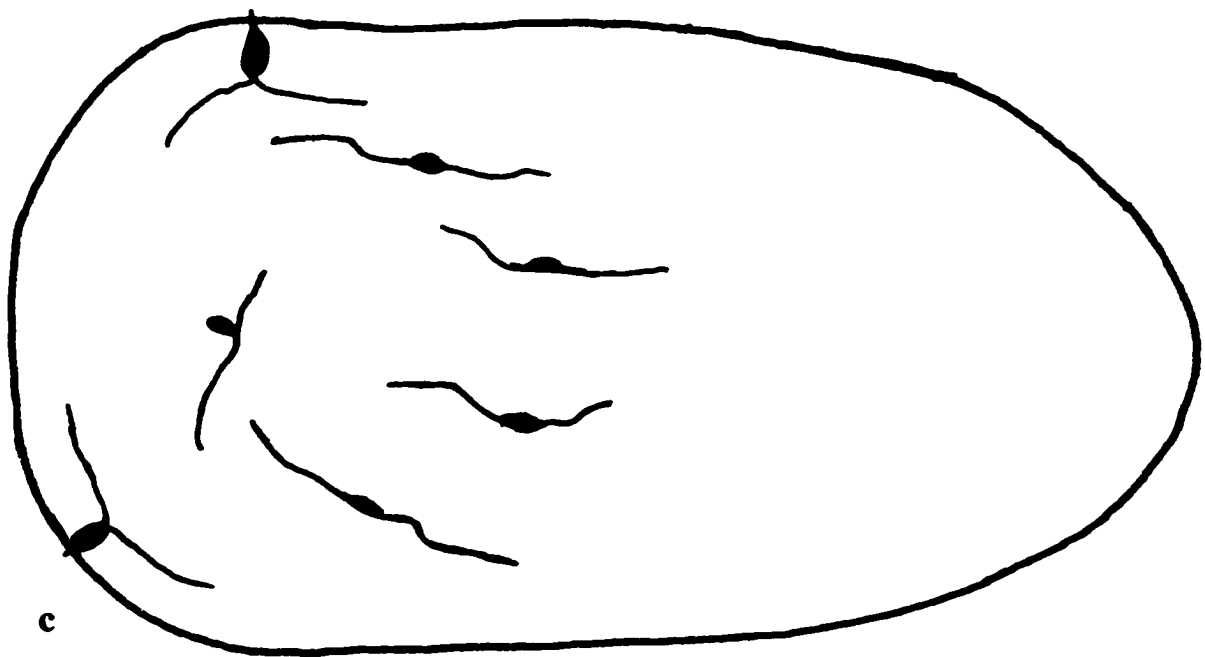
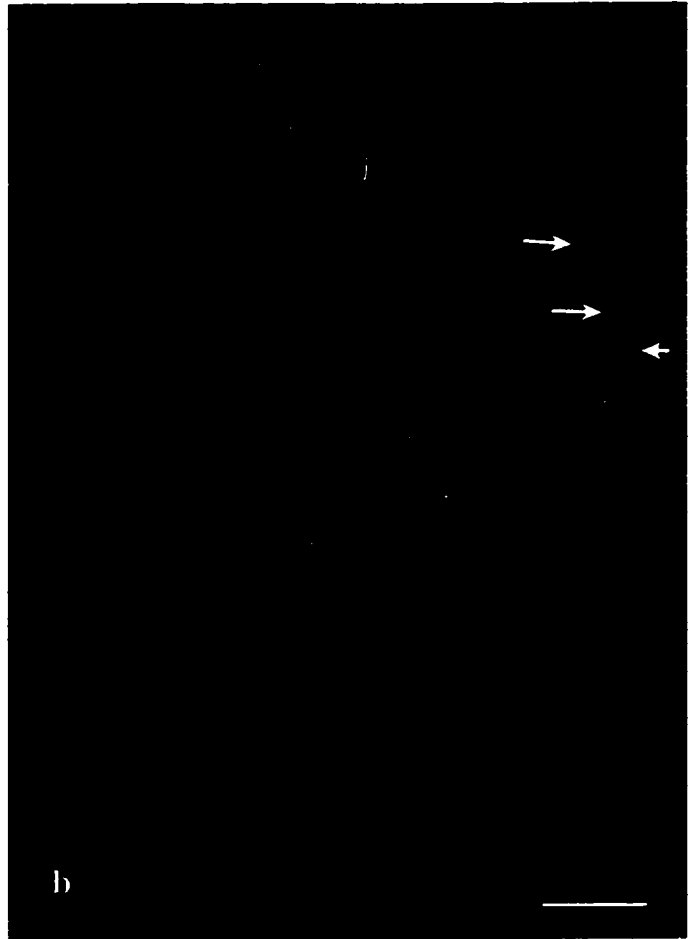
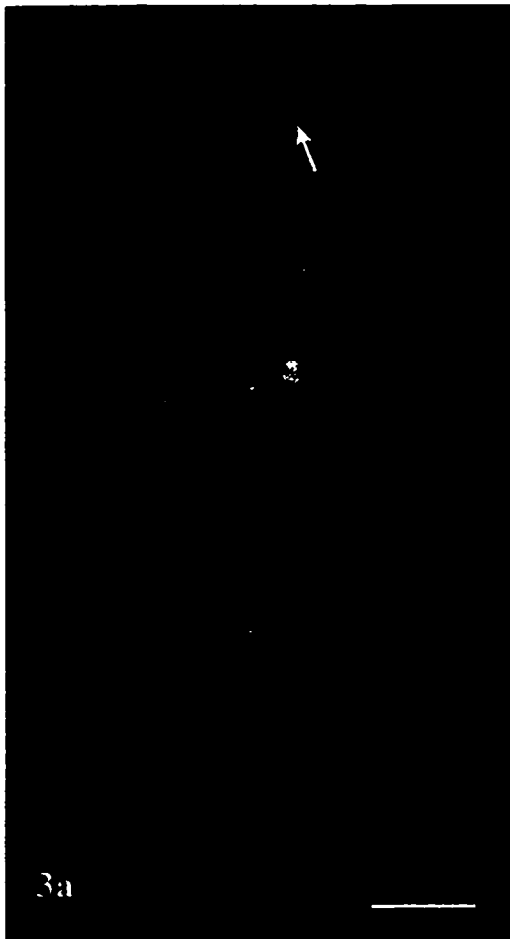
Fig. 3 FMRFamide-positive neurons in the planula

a) Sensory cell (arrowhead) and fibre (arrow) in the early planula.

b) Bipolar cells (arrowheads) and fibres (arrows) at the anterior end.

Scale bar for a) and b) = 10 μ m

c) Diagrammatic representation of distribution of FMRFamide-positive cells in the planula



arranged at more or less the same level (Fig. 4e), towards or in the basal parts of the supportive cells, which are columnar in shape (Fig. 5a). Ectodermal cells also frequently contain large inclusions in their basal parts that are filled with an amorphous substance (Fig. 5a,b). Occasionally, nerve fibres can be seen at the base of the ectodermal layer (Fig. 5c), flanked by supportive cells or, though rarely, by extensions from a single cell. Dense-cored vesicles are present in the profile of some fibres (Fig. 5c, d), and appear throughout the length of the fibres. Nerve as well as sensory cells contain clear vesicles, a well developed Golgi apparatus, ribosomes and mitochondria. Nematocysts are frequently interspersed with the ectodermal cells.

The endodermal cells lack a columnar arrangement and their shape is more irregular due to interdigitations of the membranes with those of their neighbours (Fig. 5a, b). The cells contain vacuoles that are larger than those in the ectodermal cells, and the only prominent inclusions are filled with an amorphous substance similar to some of those of the ectoderm. Sometimes, the inclusions contain spherical and ribbon-like electron-dense material (Fig. 5a).

Induction of Metamorphosis

Variable conditions of the seawater system and in the seawater tables led to the loss of several experimental set-ups. A protozoan infection caused the death of the planulae in several cultures, and twice in as many weeks, bursting neighbouring tanks caused the destruction of experimental set-ups. Also, due to a long-lasting problem with gas supersaturation of the seawater, most animals, including the adult females, were badly damaged to the point of being torn apart. Altogether, the experiment or parts of it had to be repeated 17 times. Under the circumstances, I attempted to have 5 replicates with 50 planulae each for each of the treatments and controls, and to follow the development of the planulae for at least ten days.

Fig. 4 Electron micrographs of ectodermal cells in the planula

a) Cross section through ectoderm; many supportive cells appear square in cross section and contain a variety of inclusions. Each cell projects a cilium (arrow) and microvilli from its apical surface; arrowhead points to microvillus.

Scale bar = $1\mu\text{m}$

b) Section of a) at higher magnification; large inclusions appear to be filled to varying degrees with a mucus-like substance (arrow), or contain electron-lucent material (double arrow). Smaller, electron-dense granules are also found (curved arrow). At top: cilium and microvilli. Scale bar = $0.5\mu\text{m}$

c) Arrangement of membranes (arrow), and d) dense-cored vesicles (dcv) and electron-dense granule (arrow). Scale bar for c) and d) = $0.5\mu\text{m}$

e) Level of nuclei (n) towards base of cells; (nu) nucleolus. Scale bar = $1\mu\text{m}$

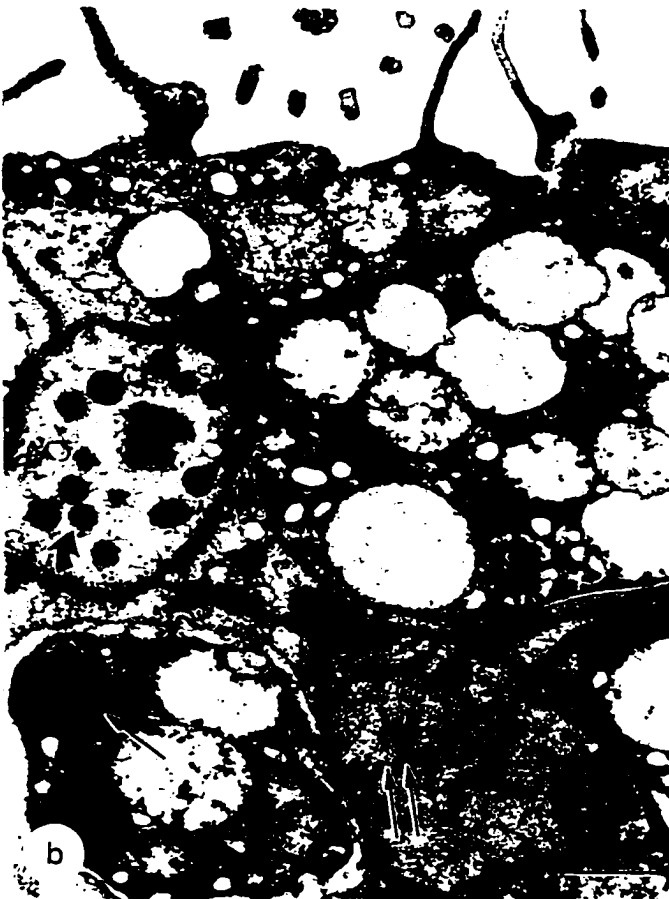
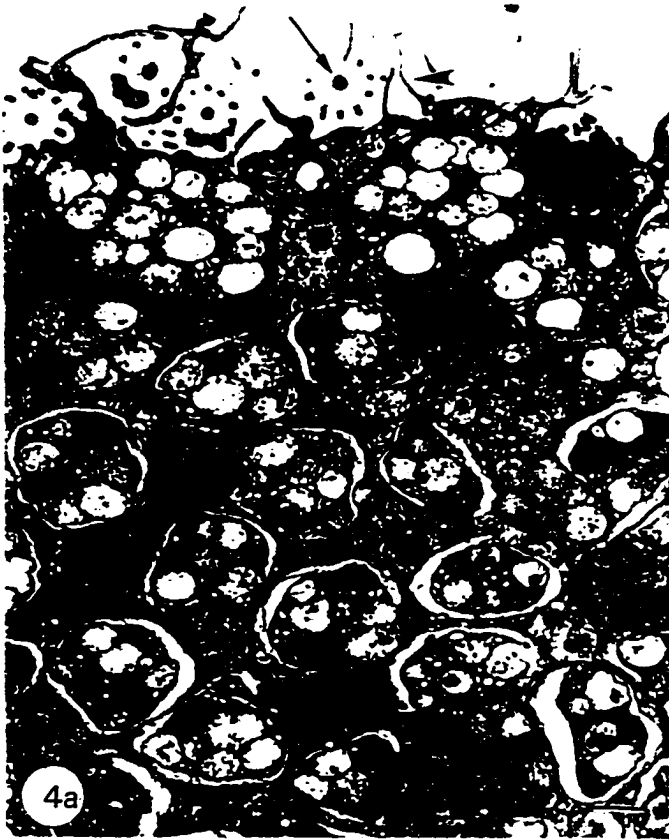
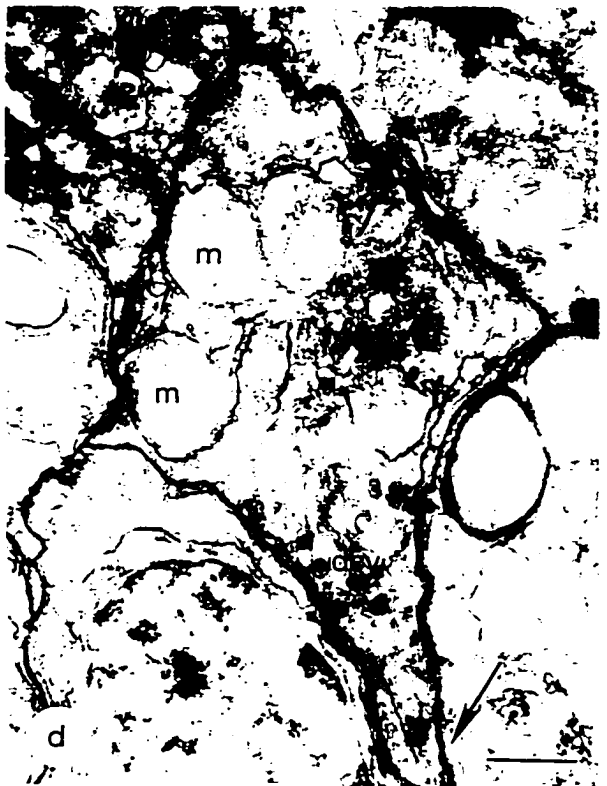


Fig. 5 Electron micrographs of cells in the planula

- a) Ectodermal cells (EC) are columnar in shape and are separated by a thin mesogloea (arrow) from the endodermal cells. Both cell types possess inclusions filled with an amorphous substance (arrowhead). In the endoderm, the latter can contain electron-dense spherical and ribbon-like structures (double arrow); n: nucleus. Scale bar = $1\mu\text{m}$
- b) Endodermal cells with large vacuoles; membranes of neighbouring cells interdigitate (arrow). Scale bar = $1\mu\text{m}$
- c) Neuronal processes at base of ectoderm containing dense-cored vesicles (dcv). Scale bar = $0.5\mu\text{m}$
- d) Part of soma of neuron with dense-cored vesicles (dcv) and mitochondria (m), and extending process (arrow). Scale bar = $0.5\mu\text{m}$.



In a preliminary experiment, 68% of the planulae in FSW were undergoing metamorphosis after 10 days, and none of the controls showed a significant difference in the rate of metamorphosis compared to seawater alone: H₂O and NRA: 70% ($0.344 < P < 1$), and 1% DMSO: 73% ($P=0.344$). The rate of metamorphosis was either significantly enhanced or reduced by the treatments: in T₄: 91% ($P=0.002$) of the planulae had undergone metamorphosis after 10 days, in I₂: 95% ($P=0.002$), in FMRFamide: 47% ($P=0.021$), in I₂ plus FMRFamide: 78% ($P=0.021$), in anti-FMRFamide: 85% ($P=0.021$), and in MgCl₂: 0% ($P=0.002$) (Table 1, Fig. 6). While none of the planulae in MgCl₂ metamorphosed, some did attach, but not permanently. At the end of the experiment, the animals were unchanged in their appearance and behaviour, and remained so for several weeks if left in the treatment. The addition of iodine and thyroxine to the MgCl₂ treatment also did not induce metamorphosis in any of the planulae. Once removed to seawater, they commenced to settle and metamorphose at a rate comparable to that in seawater, *i.e.* 62% were undergoing metamorphosis after 10 days ($P=0.754$).

In a second attempt at this experiment, 92% and 91.5% of the planulae metamorphosed in $1 * 10^{-7}$ M thyroxine and $1 * 10^{-7}$ M iodine, respectively, as did 32.5% in $1 * 10^{-7}$ M FMRFamide ($0.002 < P < 0.021$), but none did in 0.13M MgCl₂, compared to 66% in FSW (Table 2, Fig. 7).

Table 1 Induction of metamorphosis in planulae

5*50 planulae were exposed to each of the treatments for 10 days. Each datum in the table represents the average of 5 trials. The planulae were examined every day for signs of metamorphosis, such as broadening of the oral pole, and development of the mouth and the tentacle buds. For each day, the data represent the average number of planulae undergoing metamorphosis.

Days	FSW	H ₂ O (1:100)	NRA (1:200)	DMSO (1%)	T ₄ (10 ⁻⁴ M)	I ₂ (10 ⁻⁵ M)	FMRFa (2*10 ⁻⁸ M)	I ₂ (10 ⁻⁵ M) + FMRFa (2*10 ⁻⁸ M)	anti- FMRFa (1:200)	MgCl ₂ (0.13M)	MgCl ₂ (0.13M) followed by FSW
1	0.6	0	0	0	16.6	10	0.4	6	0	0	7
2	3.6	7	1.6	6	25	16	3.8	15	5	0	14
3	8.8	7	6.6	8.6	37.6	26	5	22	19	0	23
4	22.2	23	21	17.6	37.6	30	6.6	23	32.6	0	23
5	27	26	29.6	27.6	41	36	8.8	28	35.6	0	23
6	27.2	26	31	31.6	41	42	9	23	36	0	24
7	31	29	31.6	32.6	42	45	11.4	37	36	0	25
8	31.8	31	32.6	33	42.6	44.6	19	35	39	0	28
9	33.6	32	33	34	42.6	47.6	19.8	36	39	0	28
10	34	35	35	36.6	45.6	47.6	23.6	39	42.6	0	31
%*	68	70	70	73	91	95	47	78	85	0	62
p (from sign test)		0.344	1	0.344	0.002	0.002	0.021	0.021	0.021	0.002	0.754

* Percent animals undergoing metamorphosis after 10 days

Fig. 6 Induction of metamorphosis in planulae of *Aurelia aurita*

5*50 planulae were exposed to each of the treatments for 10 days and were monitored daily for signs of metamorphosis, such as settlement, broadening of the oral pole, and development of tentacle buds.

The treatments consisted of thyroxine (T_4) ($10^{-4}M$), iodine (I_2) ($10^{-5}M$), FMRFamide ($2 * 10^{-8}M$), iodine ($10^{-5}M$) plus FMRFamide ($2 * 10^{-8}M$), anti-FMRFamide antibodies, diluted 1:200 in 1% DMSO in seawater, and $MgCl_2$ ($0.13M$). The controls consisted of FSW, H_2O (1:100), a non-related antibody (NRA) (1:200), and 1% DMSO.

After 10 days, the average percent of animals undergoing metamorphosis was calculated from the five trials for each of the treatments.

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

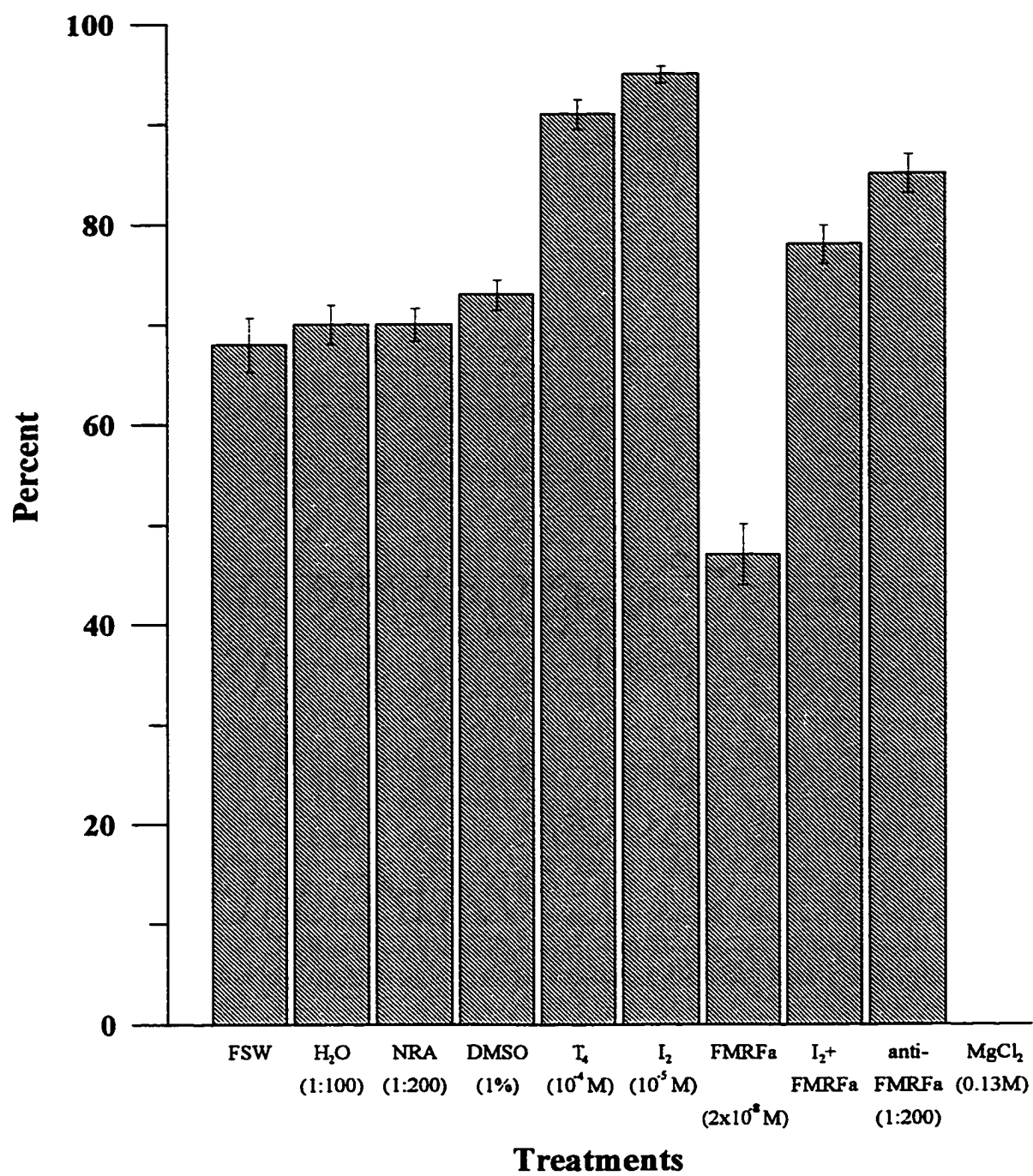


Fig. 6 Effects of treatment on induction of metamorphosis in planulae of *Aurelia aurita*

Table 2 Effects of treatments (thyroxine, iodine, FMRFamide, and $MgCl_2$) on induction of metamorphosis in planulae

For each treatment, 5*50 planulae were used and observed for 10 days for signs of metamorphosis, such broadening of the oral pole, and development of the mouth and the tentacle buds. Each datum represents the average number (from the 5 trials) of planulae undergoing metamorphosis.

Days	FSW	T ₄ (10 ⁻⁷ M)	I ₂ (10 ⁻⁷ M)	FMRFamide (10 ⁻⁷ M)	MgCl ₂ (0.13M)
1	0.6	11.2	6.6	0.4	0
2	3.6	22	14.8	2.6	0
3	8.8	30.8	22.8	3.2	0
4	20.4	37.6	30.6	5	0
5	27.2	39.6	34.8	6.6	0
6	28	45	43.2	6.8	0
7	29.8	45	44.8	8.4	0
8	30.2	45.6	44.8	11	0
9	33	46	45.8	16	0
10	33	46	45.8	16.2	0
%*	66	92	91.5	32.5	0
p (from sign test)		0.002	0.002	0.002	0.002

* Percent animals undergoing metamorphosis after 10 days

Fig. 7 Effects of treatments (thyroxine, iodine, FMRFamide, and MgCl_2) on induction of metamorphosis in *Aurelia aurita* planulae

Per treatment, 5*50 planulae were used and monitored for 10 days for signs of metamorphosis, such as settlement, broadening of the oral pole, and development of tentacle buds. The planulae were exposed to thyroxine (T_4), iodine (I_2) and FMRFamide (FMRFa), all at concentrations of 10^{-7}M , as well as 0.13M MgCl_2 . The control consisted of filtered seawater (FSW).

The standard errors of the means (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

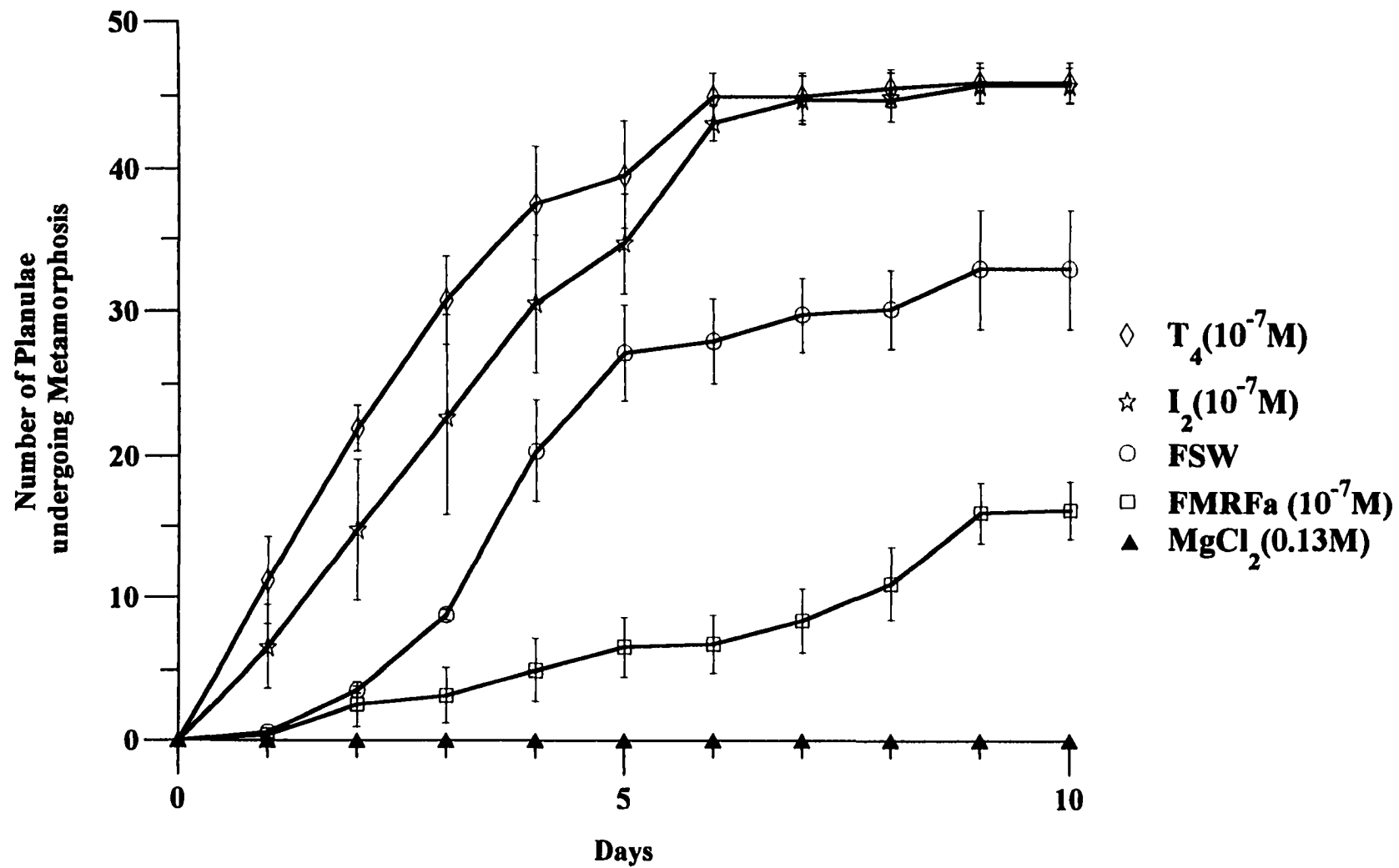


Fig. 7 Induction of metamorphosis in *Aurelia aurita* planulae

Some of the treatments had different effects on metamorphosis compared to attachment of the planulae. Since attachment does not have to be permanent, fewer planulae can be attached in one day compared to the previous one. Larvae were considered attached as long as they remained so during the count, and only the final numbers (day 10) were used for comparison of the treatments. In FSW, 73.2% of the planulae were attached, compared to 89% and 93.5% in $1 \times 10^{-7}\text{M}$ thyroxine and iodine ($P=0.002$), respectively, anticipating their effect on metamorphosis. However, 100% of the larvae in $1 \times 10^{-7}\text{M}$ FMRFamide ($P=0.002$) were attached (only 32.5% of which metamorphosed). While some of the planulae in 0.13M MgCl_2 were attached during the experiment, none remained so by day 10 (Table 3, Fig. 8)

Table 3 Effects of treatments (thyroxine, iodine, FMRFamide, and MgCl_2) on attachment of planulae

5*50 planulae were exposed to each of the treatments for 10 days. Animals were monitored daily, and were counted as attached as long as they remained so for the duration of the count. Each datum represents the average of 5 trials.

Days	FSW	T_4 (10^{-7}M)	I_2 (10^{-7}M)	FMRFamide (10^{-7}M)	MgCl_2 (0.13M)
1	3.6	34	20.2	18.6	1
2	14.4	37.6	37	21.8	2
3	29.6	43.8	42.8	24.6	4.6
4	31.6	40.6	46.6	20	4.6
5	36.6	45.8	45.2	27.2	1.8
6	34.8	46	46	27.6	0.2
7	35.8	45	46	31.2	0
8	36.6	43.8	46	36.4	0
9	36.6	44.6	46.8	36.8	0
10	36.6	44.6	44	36.8	0
%*	73.2	89	93.5	100	0
p (from sign test)		0.002	0.002	0.344	0.002

* Percent animals attached on day 10

Fig. 8 Effects of treatments (thyroxine, iodine, FMRFamide, and $MgCl_2$) on attachment of *Aurelia aurita* planulae

Per treatment, 5*50 planulae were used and observed for attachment, even temporary, for 10 days. The planulae were exposed to FMRFamide, iodine and thyroxine, all at concentrations of $10^{-7}M$, as well as to $0.13M$ $MgCl_2$. The control consisted of filtered seawater (FSW).

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

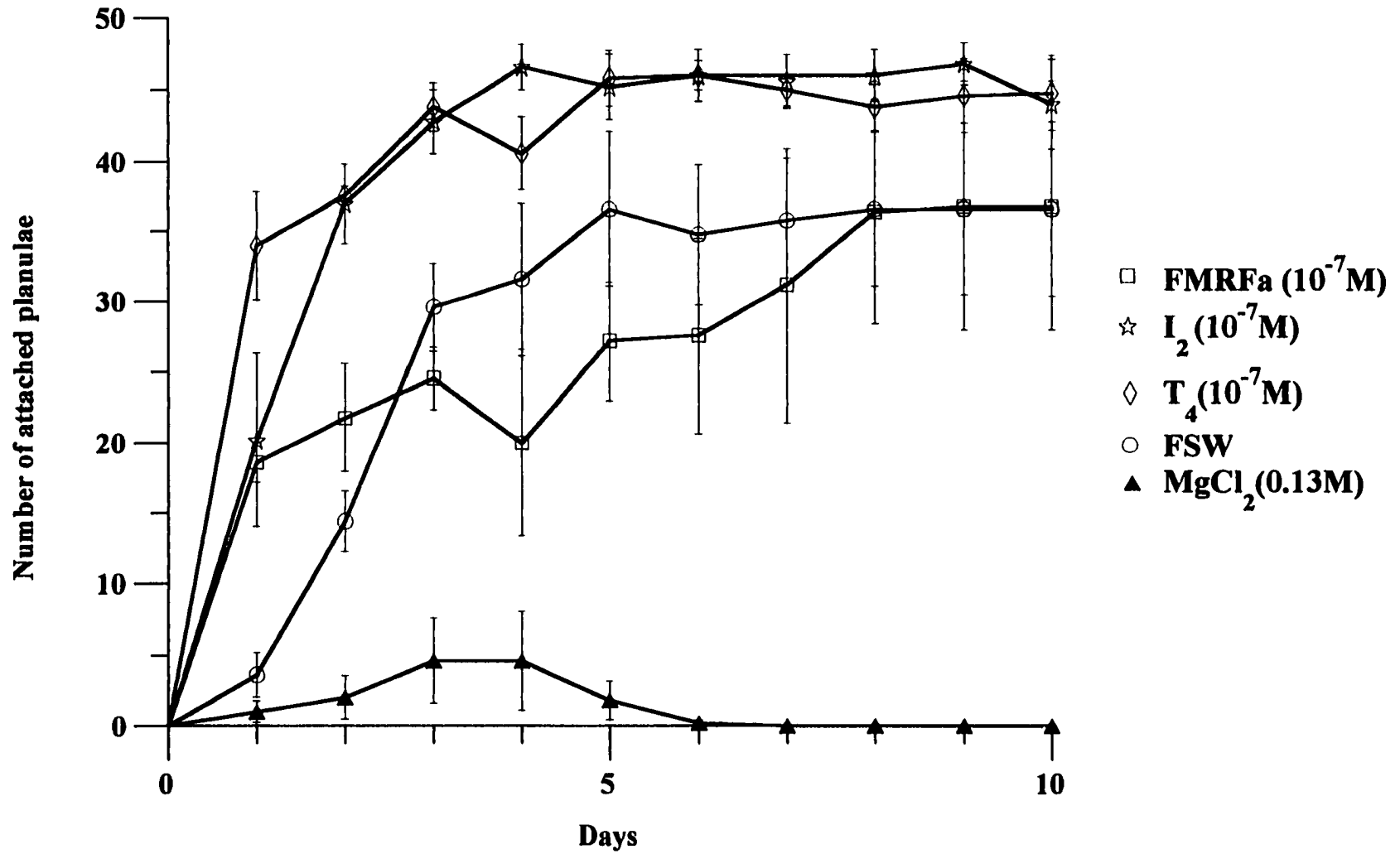


Fig. 8 Effect of treatments on attachment of *Aurelia aurita* planulae

Due to the conditions of the sea water systems referred to earlier, the experiments using various concentrations of retinoic acid as an inducer of metamorphosis had to be repeated eight times, and I was only able to monitor the set-ups using 10^{-6}M and 10^{-7}M retinoic acid for at least 10 days (Table 4, Fig. 9). The latter seem therefore the most reliable for the assessment of the effect of RA. After 10 days, 63.6% ($P=0.109$) of animals in 10^{-6}M RA, and 70% ($P=0.002$) of animals in 10^{-7}M RA were undergoing metamorphosis, compared to 50% in FSW and 56% ($P=0.109$) in ETOH. While the other experiments using RA are incomplete, it is of interest to note that in one of the failed trials, using 10^{-5}M RA, in which the planulae remained for several days, they started to die.

Table 4 Induction of metamorphosis with retinoic acid

5*50 planulae were exposed to each of the treatments for 10 days. The planulae were monitored daily for signs of metamorphosis. Each datum represents the average of 5 trials.

Days	FSW	ETOH (0.1%)	RA (10^{-6}M)	RA (10^{-7}M)
1	5.2	2.6	4.6	9
2	9.8	3.2	13.4	13.4
3	11.8	5.2	16.6	17
4	14.6	6.8	16.6	22.8
5	17.4	9.2	16.6	24.6
6	17.4	9.2	27.4	24.6
7	22.8	20	27.4	33
8	22.8	20	30.2	33
9	24	26	31.2	34.6
10	25	28	31.8	35
%*	50	56	63.6	70
p (from sign test)		0.109	0.109	0.002

* Percent animals undergoing metamorphosis after 10 days

Fig. 9 Effect of retinoic acid on metamorphosis in planulae of *Aurelia aurita*

Per treatment, 5*50 planulae were used and observed for 10 days. Treatments consisted of 10^{-7} M and 10^{-6} M retinoic acid (RA), and the controls consisted of filtered seawater (FSW) and 0.1% ETOH. The latter had been used to originally dissolve the retinoic acid.

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

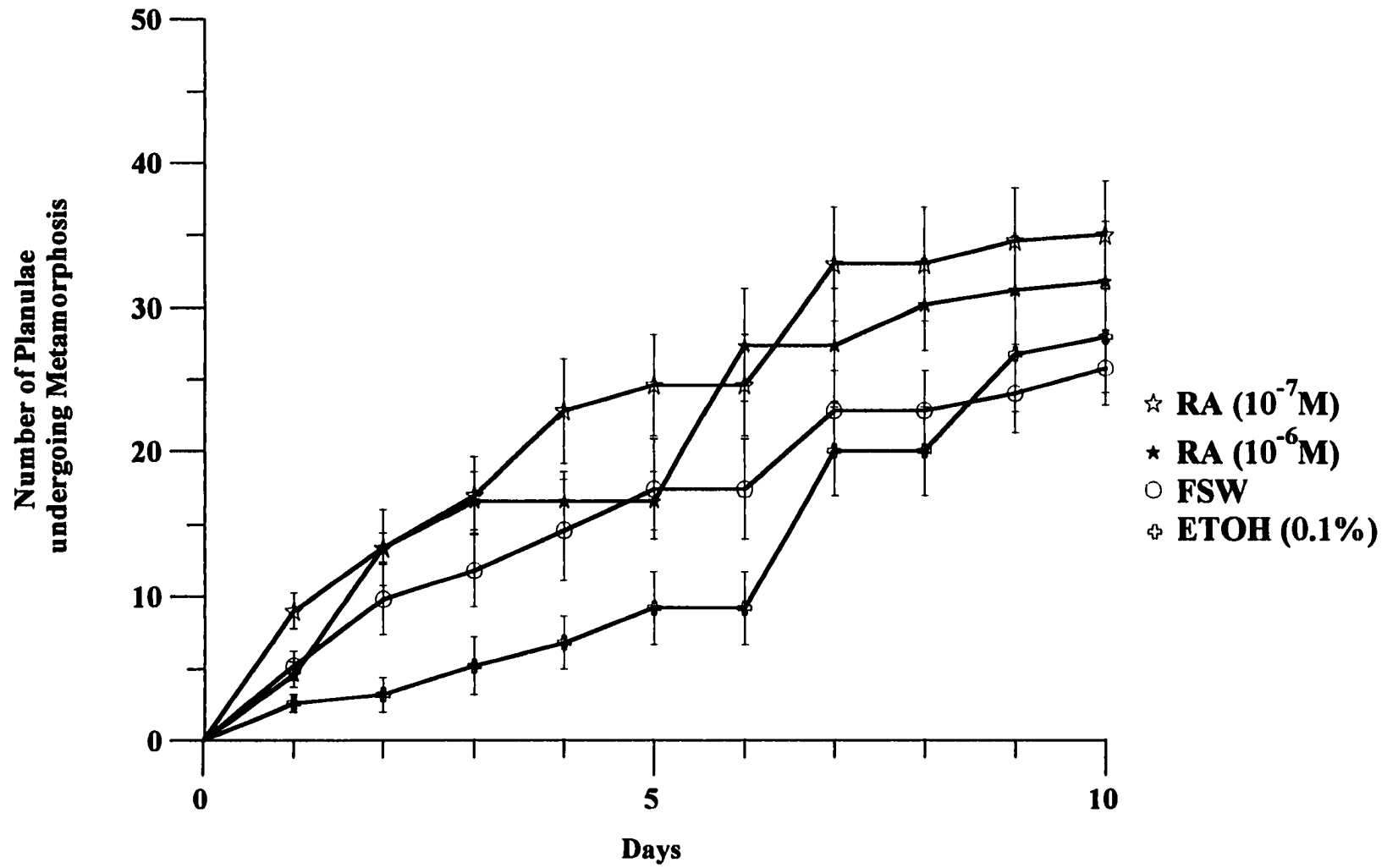


Fig. 9 Effect of retinoic acid on metamorphosis of planulae of *Aurelia aurita*

When the planulae were cut in half, out of 10 animals (20 halves), 8.5 animals (42.5%) of those in FSW, and 8 (40%) of those in thyroxine (10^{-7}M) were undergoing metamorphosis after 10 days (Table 5). There was no significant difference in the effects of the treatment and the control ($P=1$).

Table 5 Effect of thyroxine on metamorphosis of operated animals

5*10 planulae were cut into posterior and anterior halves each for the treatment, thyroxine (10^{-7}M), and the control of FSW. Animals were monitored daily for signs of metamorphosis. Each datum represents the average of 5 trials.

Days	FSW	T_4 (10^{-7}M)
1	0	0
2	1	2
3	1	2
4	1	2
5	1.4	2
6	6.6	5
7	6.6	5.6
8	8.2	7.2
9	8.4	7.4
10	8.6	8
%*	43	40
P (from sign test)		1

* Percent animals undergoing metamorphosis after 10 days.

Discussion

Laboratory observations of the behaviour of *Aurelia aurita* planulae correspond to those reported in the literature as far as manner of locomotion (Widersten, 1968), and preferential orientation for attachment (Brewer, 1978) are concerned. The larvae move by anti-clockwise rotation around their longitudinal axis (when viewed from their

posterior pole) due to ciliary movements. Planulae preferentially hang upside down, but also attach to the bottom of the dish. Larval settlement and metamorphosis on the sides of the dish are rare. As polyps in the field are also often encountered near the water surface with their oral pole pointing downward, Cargo (1979) and others have suggested that the selective settling behaviour of the planulae serves as a survival mechanism for several reasons. Animals suspended from the underside of a substrate remain unharmed from siltation which causes mortalities on the upper surface of the same substrate, are potentially more protected from overgrowth by other organisms, and could be in a more advantageous position for the release of the ephyrae during strobilation (Brewer, 1976). Brewer (1976) also suggested that attachment to the hyponeuston may be an artifact due to laboratory culture. In the experiments reported here, *Aurelia* planulae did prefer to attach to the hyponeuston, but also settled and metamorphosed on the bottom of the dish in greater numbers than on its sides. This is somewhat surprising, as planulae released from gravid females in a seawater tank settle and metamorphose preferably on the sides of the tank rather than the bottom, which is perhaps due to current created by the incoming water. Since polyps are also found in great numbers on the hoses feeding the water into the tank, the curvature of the inside of the beaker is an unlikely reason why more planulae do not settle on its sides.

The paddle cilia are an artifact due to stress or fixation procedures (Short and Tamm, 1991) resulting in a coiling of the axoneme in the stretched distal membrane of the cilium. The cilia in the planulae of *Aurelia aurita* appeared to have straight tips when first viewed under the light microscope, but disc-shaped tips were soon visible and were perhaps due to stress from exposure to the heat from the light source. The presence of a collar of microvilli was also observed by Lyons (1973) in a coral planula, where, according to her, they surround a flagellum rather than a cilium. She holds the distinction to be important as far as a potential metazoan ancestor is concerned, given that such an ancestor would have given rise to a ciliated acoel. Lyons (1973), having

identified the existence of a flagellate collar in coelenterates, therefore questions the probability of a planula-like ancestor for the metazoa. However, Widersten (1968) examined a number of anthozoan planulae and reported that they swam by ciliary movement. Furthermore, the main distinction between the two, perhaps apart from their length, lies in the different manner of movement, with cilia beating and flagella moving in sinusoidal waves, which cannot be determined by ultrastructural observation alone.

Comparison of the ultrastructural morphology of the planulae of *Aurelia* with that of the scyphozoan *Cassiopea xamachana* (Martin and Chia, 1982) shows that in both, the ectodermal cells are columnar in shape and contain several types of inclusion, both have nematocysts interspersed with the ectodermal cells, and the ecto- and endodermal layers are separated by a thin mesogloea. However, *Aurelia* planulae appear to possess a greater variety of inclusions, and the nuclei are found either right at the base or towards the basal pole of the cells, rather than being located centrally, as they are in *Cassiopea*. The nature of the contents of the inclusions is unknown, but if the larger inclusions in the apical region indeed contain a mucus-like substance, they could be in various stages of releasing their contents, which would explain the difference in electron density. Unlike the columnar arrangement of the endodermal cells in *Cassiopea*, the endodermal cells in *Aurelia* are shaped more irregularly and have interdigitating membranes. The major difference in the two planulae, however, is the presence of a nervous system in *Aurelia* and the absence of the latter in *Cassiopea*.

Neither of the two scyphozoan planulae possess an apical tuft, which is present in the sea anemone *Anthopleura elegantissima* (Chia and Koss, 1979), and compared to either anthozoan or hydrozoan planulae, they are smaller and morphologically simpler in that they possess fewer types of cells. Anthozoan planulae can contain up to 15 different cell types, including three types of glandular cells in the ectoderm and 6 types of endodermal cells, as is the case for *Anthopleura* (Chia and Koss, 1979), whereas

hydrozoan planulae such as *Mitrocomella polydiatemata* (Martin *et al.*, 1983) and *Pennaria tiarella* (Martin and Thomas, 1980) have seven types of ectodermal and two types of endodermal cells. As far as the nervous system of the planulae of *Aurelia* is concerned, it resembles most closely that of the hydrozoan planulae, which also possess ectodermal sensory cells and neurons. The nervous system of *Anthopleura* is more complex in that it consists of an apical organ, two types of ectodermal and one type of endodermal receptor cells, and central neurons.

The factors associated with and inducing metamorphosis have been more thoroughly studied in the Hydrozoa than in the Scyphozoa. In the marine hydroid *Hydractinia echinata*, it has been shown that protein synthesis is necessary for switching off the pattern-forming system of the larva and switching on that of the polyp (Kroiher *et al.*, 1991). Various agents have been reported to induce metamorphosis in this animal, including CsCl, NH_4^+ , the marine bacterium *Alteromonas espejiana*, and heat shock. In Scyphozoa, the most thorough study of induction of metamorphosis is on *Cassiopea*. Planulae of *Cassiopea andromeda* do not metamorphose in sterile artificial seawater devoid of organic substances, but require exogenous inducers such as cholera toxin and thyrotropine (Fitt *et al.*, 1987).

In *Aurelia*, there is strong evidence that both thyroxine and iodine induce settlement and metamorphosis in planulae, as does, to a lesser degree, retinoic acid. Both of thyroxine and iodine increased the rate of metamorphosis to over 90% ($P=0.002$ for either) compared to 66% in FSW. When 10^{-6}M and 10^{-7}M RA were compared in the same trial, only 10^{-7}M RA produced results that were significantly different from those in FSW ($P=0.002$). None of the concentrations of RA tried, however, were able to induce metamorphosis at a comparable rate to thyroxine or iodine.

For an agent to enhance the rate of metamorphosis in larvae, it can either represent a stimulus that initiates metamorphosis, or it can enhance metabolic processes

associated with metamorphosis. The effect of thyroxine and retinoic acid is on gene expression, mediated by nuclear receptors that belong to the steroid/thyroid-hormone receptor superfamily (*e.g.* Zhang *et al.*, 1992); most likely that is how they act in *Aurelia*. Fitt *et al.* (1987) proposed that thyrotropine affects metamorphic development rather than representing a triggering mechanism in *Cassiopea*. It is conceivable that the same is true for the effect of thyroxine in *Aurelia*, which could either cause enhanced gene activity necessary for the formation of the polyp, or repress larval gene activity. While it is tempting to compare the effects of thyrotropine in *Cassiopea* with those of thyroxine in *Aurelia*, it should be noted, however, that Fitt *et al.* (1987) only provide data for 2 and 3 days, presumably because at that time, 100% of the animals were "settled". In a preliminary study (Wolk *et al.*, 1985), they described settled animals as having "no or reduced tentacles". It is therefore possible that at least some of their animals were temporarily attached, and as has been seen in *Aurelia*, the rate of attachment does not necessarily correlate with the rate of metamorphosis.

Fitt *et al.* (1987) also proposed that the failure of the planulae to complete metamorphosis in sterile artificial seawater and their requirement for exogenous inducers stems from the fact that the larvae may have evolved in an environment with an exogenous inducer that, through evolutionary time, replaced or suppressed the endogenous mechanism of triggering metamorphosis. However, although the planulae do not possess a mouth and do not feed by predation, many cnidarians are able to directly absorb dissolved organic material through the ectoderm (Schlichter, 1982), so that the failure of the planulae to metamorphose in seawater devoid of any organic compounds could at least partially be due to the effects of starvation. Furthermore, Spangenberg (1974) reported that *Aurelia* scyphistomae have the ability to produce thyroxine, and also secrete iodocompounds into the surrounding medium. If the planulae, too, can produce thyroxine, the addition of iodine to the seawater would provide the planulae with an increased supply of iodine compared to seawater alone,

and thus with a necessary building block for the formation of thyroxine (=tetraiodothyronine). If the planulae also have the ability to secrete iodocompounds, it would provide an explanation as to why the larvae are frequently encountered in clumps at various stages of metamorphosis. Once a suitable substrate has been found by some planulae, others would be encouraged to settle at this location, also.

As far as retinoic acid is concerned, its presence or that of its receptors has not been demonstrated in coelenterates. However, the thyroid hormone receptors (TR) require auxiliary nuclear proteins for efficient DNA binding, and there is evidence that the retinoid X receptor is one of those proteins, since it enhances the transcriptional activity of TR at low retinoic acid concentrations (10^{-8}M - 10^{-7}M) by heterodimer formation (Zhang *et al.*, 1992). Whatever the mode of action of retinoic acid in *Aurelia*, it is interesting that its effect on metamorphosis in *Ciona intestinalis* (Denucé, 1991) is different. Whereas it is dose-dependent in both cases, causing malformations and larval death in *Ciona* and no significant effect on metamorphosis in *Aurelia* at concentrations of 10^{-5}M , metamorphosis is suppressed in *Ciona* but enhanced in *Aurelia* with 10^{-7}M RA. The results are doubly intriguing since there is evidence that thyroxine enhances metamorphosis (Denucé, 1991). However, both thyroxine and RA can either enhance or depress gene activity depending on the target, and perhaps the different effects of RA are simply due to different targets in the two species. However, I have also found that prolonged exposure to high concentrations of RA can lead to larval death in *Aurelia*, and given that 10^{-7}M RA had no effect on hatching in *Ciona*, it would be interesting to examine the effect of even lower doses on metamorphosis in this animal.

FMRFamide and MgCl_2 , on the other hand, reduce or inhibit metamorphosis. MgCl_2 could be involved in the reduction of Ca^{2+} ; the concentrations used (0.13M) would probably block or decrease synaptic transmission and thus reduce any neurogenic effect on metamorphosis. However, it would also affect calcium channels in other cells

and so might interfere with signal-transducing pathways that use calcium as a second messenger, such as the calcium-calmodulin second messenger system.

Perhaps FMRFamide functions as an inhibitory transmitter for neurons involved in the switch from planular to polyp pattern, or it could be excitatory for cells involved in the maintenance of the planular pattern. FMRFamide could either act as a neurotransmitter/-modulator and thus affect the actions of other neurons, or its effect could be more general. Brumwell and Martin (1996) suggest that, given that dense-cored vesicles are present along the entire length of the neurites in *Pennaria tiarella*, there could be multiple release sites for the peptide. If the vesicles were exocytized at those sites at the same time, the large amount of peptide could provide for rapid communication among all cells. They propose that RFamide is necessary for metamorphosis to occur, since larvae that have been rendered free of neurons by elimination of interstitial cells fail to metamorphose but will do so if provided with RFamide (Martin, unpublished data). Perhaps sensory cells perceive the stimulus for metamorphosis and transmit the information to ganglionic cells, which in turn convey the stimulus to the rest of the animal. It would be interesting to see those data, since they suggest a different effect of RFamide in hydrozoan and scyphozoan planulae. According to Brumwell and Martin (1996), larval RFamide-positive neurons disappear shortly after metamorphosis begins (Martin and Archer, unpublished data). This, however, could be a consequence of the onset of metamorphosis. The differences in the proposed effect of RFamide in *Pennaria* and that in *Aurelia* could be due to the age of the larvae that were used in the studies. Fitt *et al.* (1987) showed that older larvae metamorphose more readily, suggesting that age is one of the factors determining fitness to metamorphose. Perhaps FMRFamide has an inhibitory effect on metamorphosis if the larvae are not ready yet or if the proper stimulus is absent. The fact that anti-FMRFamide reverses the effect of FMRFamide suggest the removal of an inhibitory influence. The addition of iodine, as well, can overcome the latter.

As far as the experiments regarding regenerative capabilities of the larvae are concerned, although I have attempted to cut the planulae exactly in half, this is not an easy feat considering the size of the animal. I may have inadvertently damaged some parts beyond repair. Given that two processes, *i.e.* regeneration and metamorphosis are going on at the same time, it could be that this results in both of them being somewhat delayed. However, regeneration, if the planula does recover, usually occurs sooner than metamorphosis, as the halved animals swim about as before and do not appear hindered by their wound, which closes over within a few hours. While it is surprising that thyroxine did not enhance metamorphosis, it is interesting that both for FSW and thyroxine, less than half of the animals metamorphosed. Given that, at least as far as can be determined with anti-FMRFamide, the neurons are concentrated in the anterior part of the planula (the subsequent aboral pole of the scyphistoma), perhaps the posterior parts fail to metamorphose when devoid of a potential neurogenic effect. Similarly, thyroxine could be involved in the remodeling of the posterior pole of the planula into the oral pole of the polyp, which has to be regenerated first in half of the operated animals.

The Scyphistoma

Introduction

In the scyphistoma of *Aurelia aurita*, Haeckel (1881) observed ectodermal muscle cells and longitudinal muscle strands which pass through the whole length of the animal, radial and longitudinal muscle cells of the oral surface, and longitudinal muscle cells of the tentacles. According to Claus (1883), the muscle bands of the scyphistoma of *Chrysaora* were of endodermal origin and did not develop until after the second quartet of tentacles had formed (neither of which is true for the scyphistoma of *Aurelia aurita*). The muscle bands gradually disappeared in the strobila (which is true for *Aurelia*). Goette's (1887) examination of the development of *Aurelia* led him to believe that the muscle bands were not of endodermal origin, nor were they processes of epithelial muscle cells. Instead, ectodermal cells from the base of the young scyphistoma separated from the rest of the cells, and the muscle fibres developed intracellularly, surrounded by mesogloea. The fibres extended and fused towards the oral surface. Later in development, their number increased and cell boundaries became less apparent. During strobilation, the structure of the scyphistoma gradually changed into that of a medusa, *i.e.* the disc of the scyphistoma gradually and steadily developed into the ephyra. Thus, according to Goette (1887), this process only represented a division of a developing larva, a mere step in the continuous development of the medusa, and not an alteration of generations, which would require a change in form. [However, not all parts of the scyphistoma are transformed into ephyrae, and after the latter have been released, the remaining strobila reverts into a scyphistoma. Furthermore, *Aurelia* strobilae produce many ephyrae, and the oral disc of the scyphistoma only participates in the formation of the most distal of the latter.]

More recently, both the ultrastructure and strobilation have been studied in detail. In *Aurelia*, strobilation can be induced by iodine and thyroxine (Spangenberg, 1967 and 1971). Iodine also stimulates strobilation in *Chrysaora* (Black and Webb, 1973), and in both, the amount of DNA per polyp increases, while RNA and protein levels remain relatively constant (Black, 1972). In *Chrysaora*, synthesis and transport of neurosecretory material begins during the pre-strobilation growth phase and continues through early and mid-strobilation (Loeb and Hayes, 1981). Also in the latter, there is evidence that a neck-inducing factor is involved in the initiation of strobilation. This factor is water soluble and of high molecular weight, and is possibly a protein or polypeptide (Loeb, 1974).

As far as the nervous system of the scyphistoma is concerned, Chapman (1965) was unsuccessful in demonstrating neurons with either silver staining or methylene blue. At the electron microscope level, he found numerous neurites oriented parallel to the muscle fibres in the ectoderm of the tentacles and along the cord muscle, as well as a few "doubtful" neuronal profiles in the endoderm, but did not observe any sense cells, ganglion cells, or neuromuscular junctions. Also at the electron microscope level, Chia *et al.* (1984) found sensory cells in the tentacles only, and reported that a subepithelial plexus existed only throughout the tentacles, oral disc and muscle cords. A single type of neuron was associated with the muscle cells of the latter three, and the plexus consisted mostly of solitary fibres or small clusters of fibres. Chia *et al.* (1984) found no neuromuscular elements in the endoderm and the results indicated that the nervous system consisted of a single ectodermal nerve net.

I have attempted to expand on these studies by examining the nervous systems in both the scyphistoma as well as the strobila using anti-FMRamide as well as the mAb (Aa).

Results

Gross Morphology

When the planula metamorphoses into the scyphistoma, one of the first outward signs that metamorphosis takes place is the development of two tentacle buds and the mouth opening of the polyp (Fig. 10a), followed by the development of two more tentacle buds (Fig. 10b). The fully developed scyphistoma is between 2 and 5 mm in length and attaches to the substrate with the pedal disc. The body is divided into a stalk and calyx region, and the mouth is located in the center of the oral disc (Fig. 10c). Generally, 12-16 tentacles encircle the oral disc at its periphery, although different numbers of tentacles are not uncommon. The tentacles are usually unbranched, but, again, branched tentacles are encountered at times and appear to occur more commonly with an odd number of tentacles.

Internally, the gastric cavity is divided by four septa which extend from the oral disc to the junction of the calyx and stalk. Four muscle cords extend from the pedal disc to peristomal pits of the oral disc.

The first indication of strobilation is the formation of a neck in the calyx region of the polyp (Fig. 10d), followed by the development of ephyrae through transverse fission (Fig. 10e).

Light Microscopical Observations

When stained with rhodamine-phalloidin, the four muscle bands of the polyp become clearly visible (Fig. 11a). Oriented along the bands are fibres of FMRamide-positive neurons (Fig. 11b). As the bands approach the oral disc, the arrangement of fibres is funnel-shaped, with the tip of the 'funnel' on the muscle bands and its rim

Fig. 10 Gross morphology of the scyphistoma

a) and b) Electron micrographs (SEM) of early scyphistoma.

a) Indentation (arrow) forming in developing polyp; scale bar = $5\mu\text{m}$

b) Early scyphistoma with four tentacle buds (TB). Scale bar = $20\mu\text{m}$.

c) Diagram of scyphistoma. The body is divided into the calyx (C) and stalk (ST), and is attached to the substrate by the pedal disc. The scyphistoma usually has 12-16 tentacles (T) that encircle the oral disc.

d) Strobilation begins with the formation of a neck (NF) and

e) leads to the formation of a stack of developing ephyrae (E).

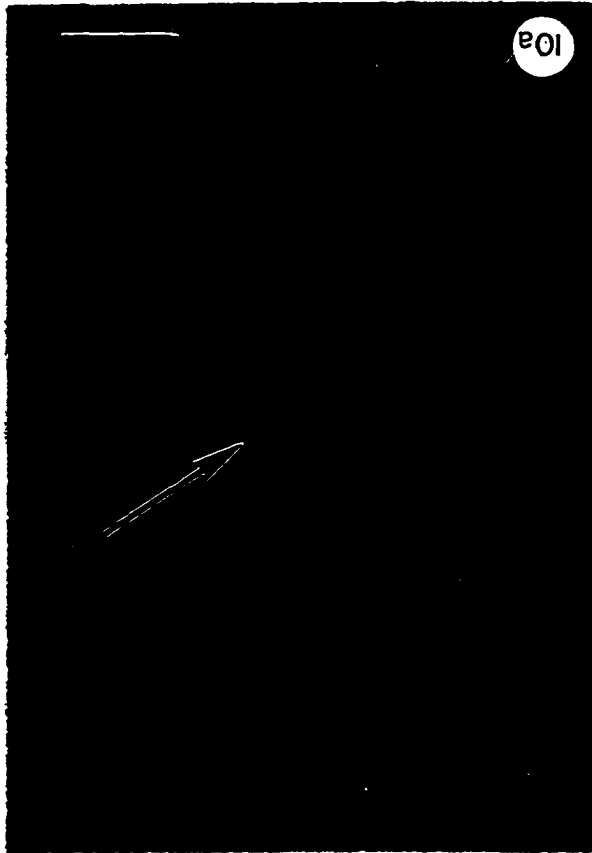
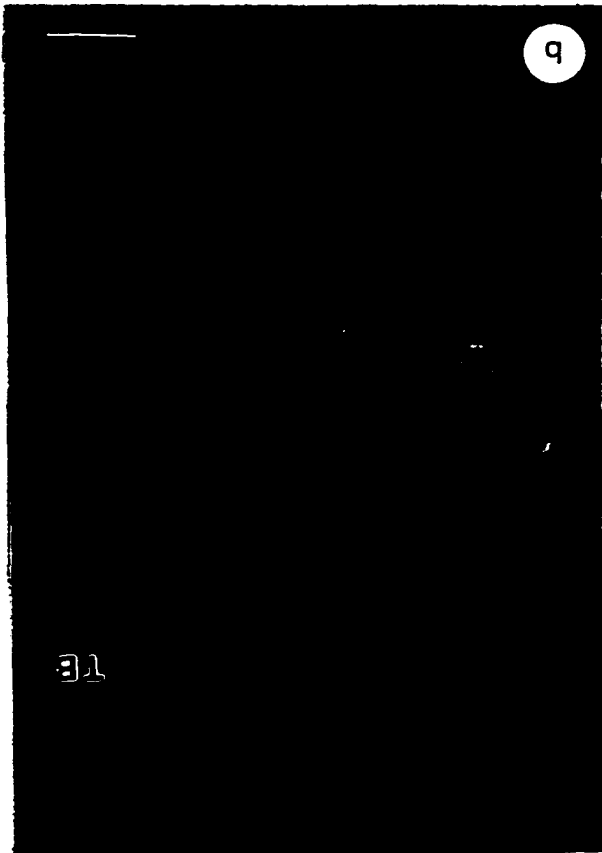
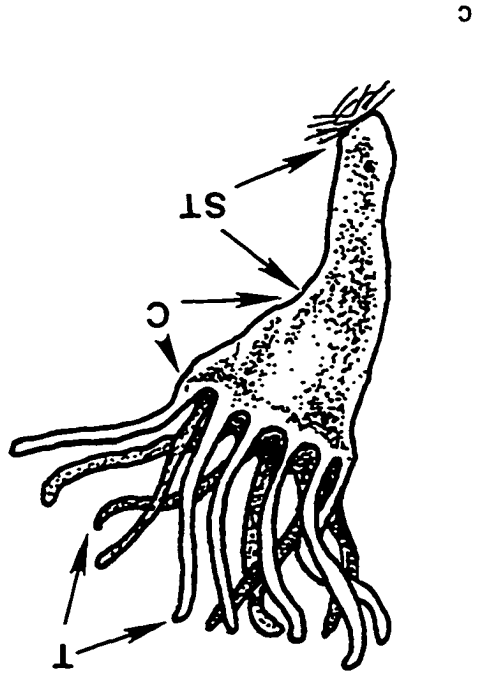
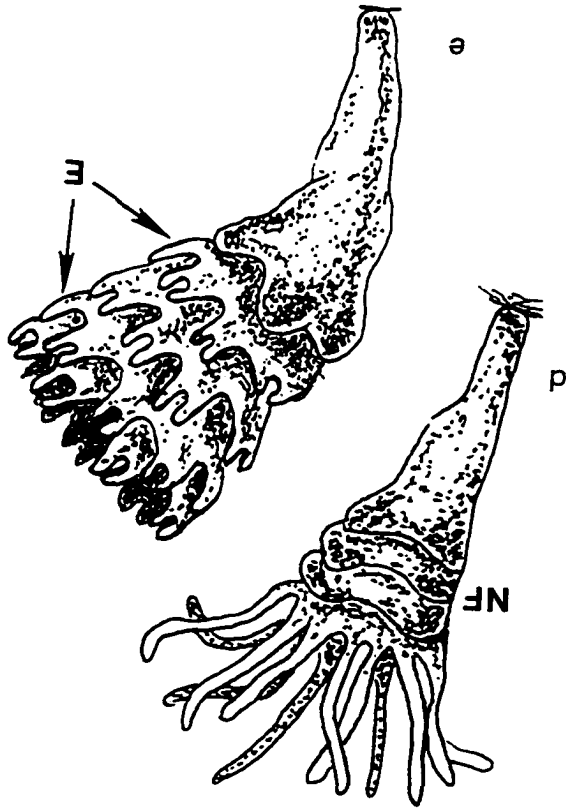
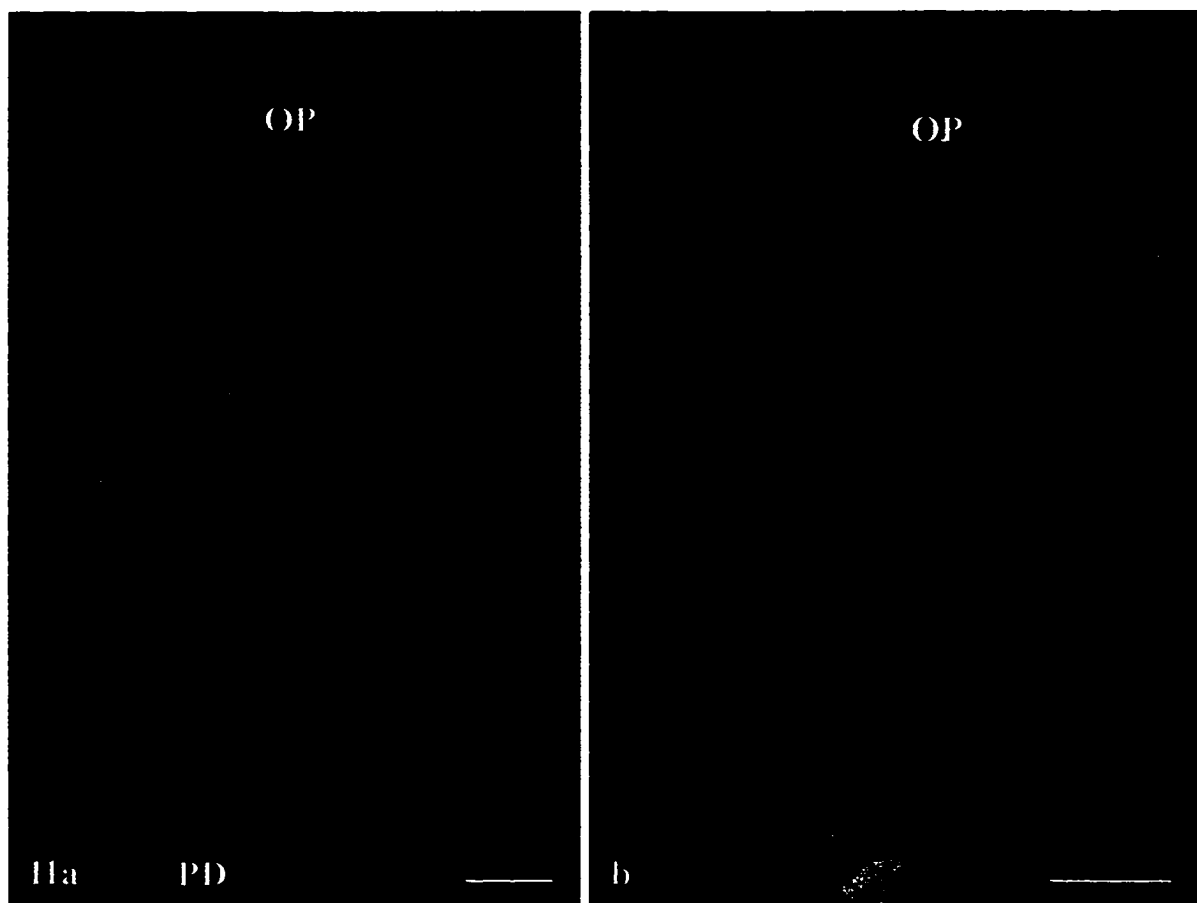


Fig. 11 Muscle bands and associated neuronal processes of the scyphistoma

a) Four muscle bands extend from the pedal (PD) to the oral disc; rhodamine phalloidin stain. OP: oral pole. Scale bar = 200 μ m

b) FMRFamide-positive fibres associated with the muscle bands. OP: oral pole. Scale bar = 200 μ m



forming part of the nerve net of the oral disc (Fig. 12a). The cell bodies of the fibres are found in this region. The body column of the scyphistoma has few other nervous structures. Occasionally, single or pairs of fibres can be seen traversing the column from the oral towards the pedal disc (Fig. 12b). The network of fibres and cell bodies is more extensive on the oral disc (Fig. 12c) and the tentacles (Fig. 12d). On the outside surface of the polyp, the fibres extend to just below the tentacle base (Fig. 12e).

The muscle bands of the scyphistoma are still present in the first few developing ephyrae of the strobila (Fig. 13a), but as they approach the ephyrae that are about to be released, swellings appear along their lengths (Fig. 13b), and they can no longer be detected in the distal part of the stacked ephyrae.

FMRFamide-positive neurons are present in the early ephyrae, where they form a dense net (Fig. 13c). Neurons labelled with mAb Aa are also already found, but are rare. They are absent in the ephyrae closest to the scyphistoma end of the strobila, but increase in frequency as the ephyrae develop more fully. In the mid region of the stack of ephyrae, oftentimes only fibres are labelled (Fig. 13 d), but fibres as well as cell bodies are labelled in the ephyrae that are about to be released (Fig. 13e).

Electron Microscopical Observations

An overview of a cross section of both ectodermal and endodermal cells reveals little variation in the cell contents (Fig. 14a). Both ecto- and endodermal cells can be highly vacuolated, but this is more pronounced in the endoderm, and particularly so in the tentacles (Fig. 14b). The tentacles of scyphistomae are solidly filled with endodermal cells whose membranes interdigitate with those of their neighbours. The cells have little cytoplasm but possess a prominent nucleus. The cytoplasm of the ectodermal cells can contain a number of different inclusions that can be filled with an

Fig. 12 FMRamide-positive neurons of the scyphistoma

- a) Neuronal fibres on the muscle bands (arrowhead) fan out towards the oral disc (OD). Arrow points to cell body. Scale bar = 10 μ m.
- b) 'Pair' of fibres (arrows) traversing the body column. Scale bar = 10 μ m.
- c) Nerve net on oral disc (OD); Scale bar = 10 μ m.
- d) Fibres and cell bodies (arrow) in tentacle. Scale bar = 10 μ m.
- e) Fibres extend to just below the tentacle base (B) on the outside surface of the scyphistoma. Scale bar = 10 μ m.

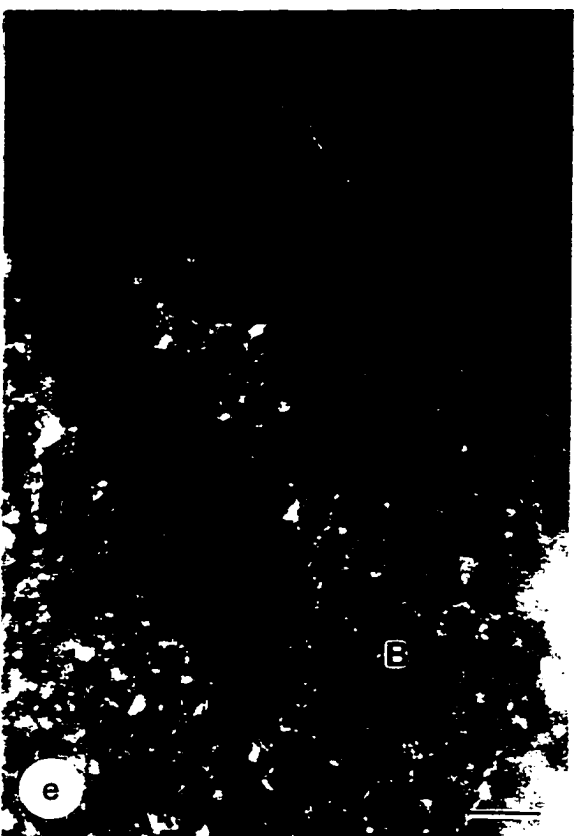
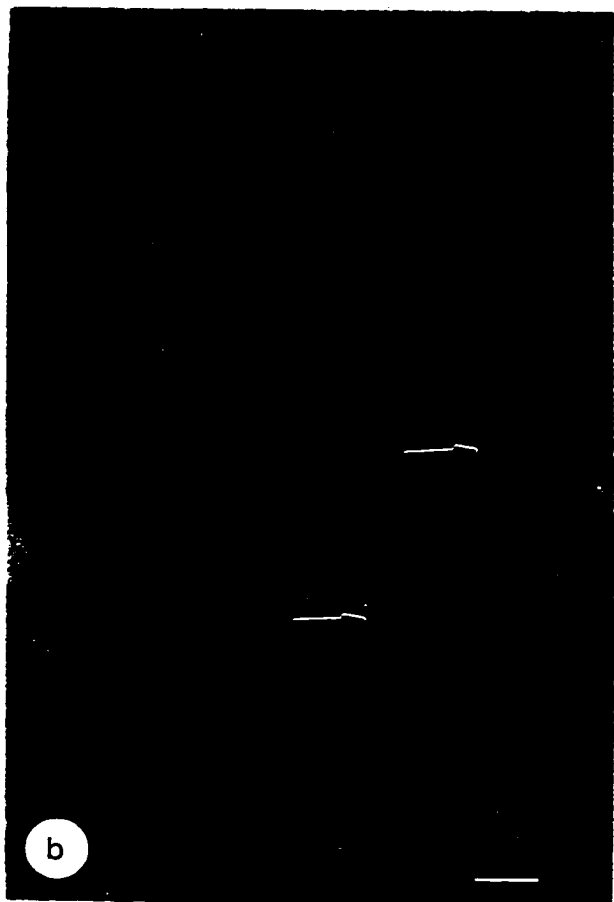
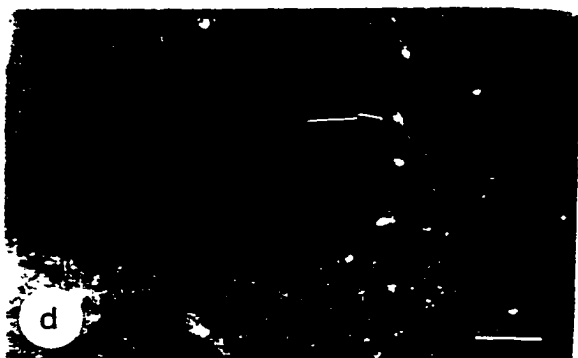
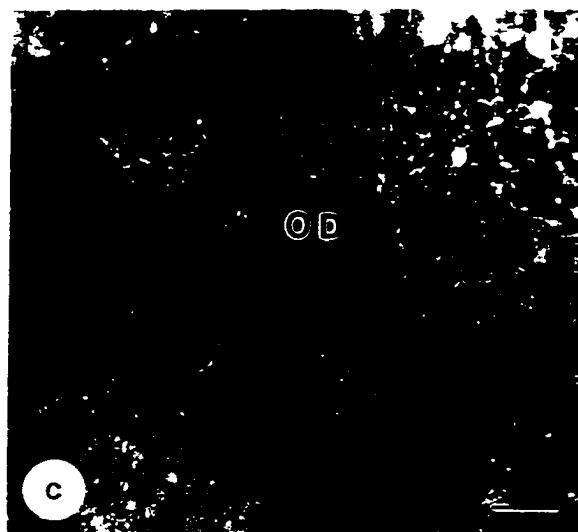
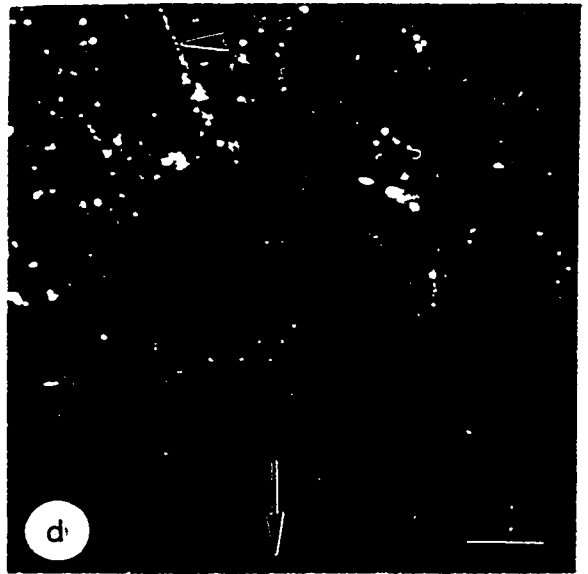
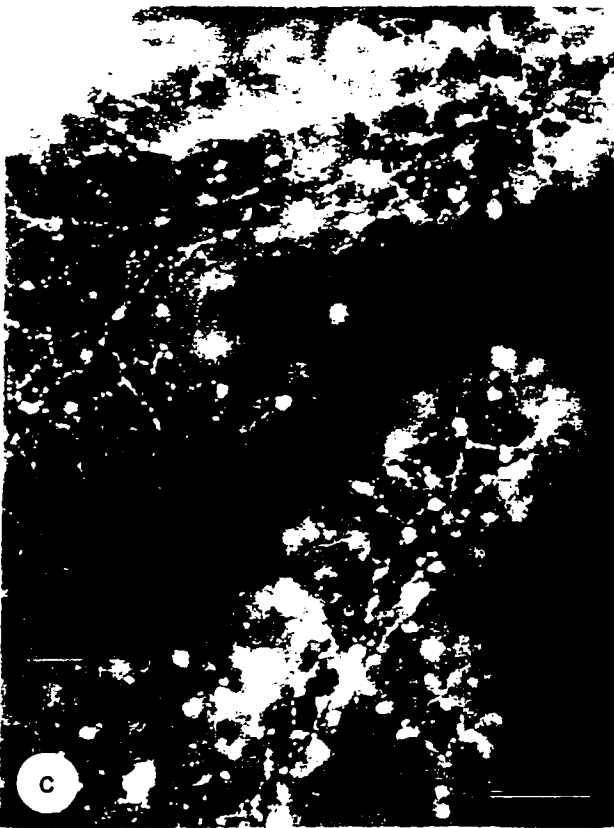
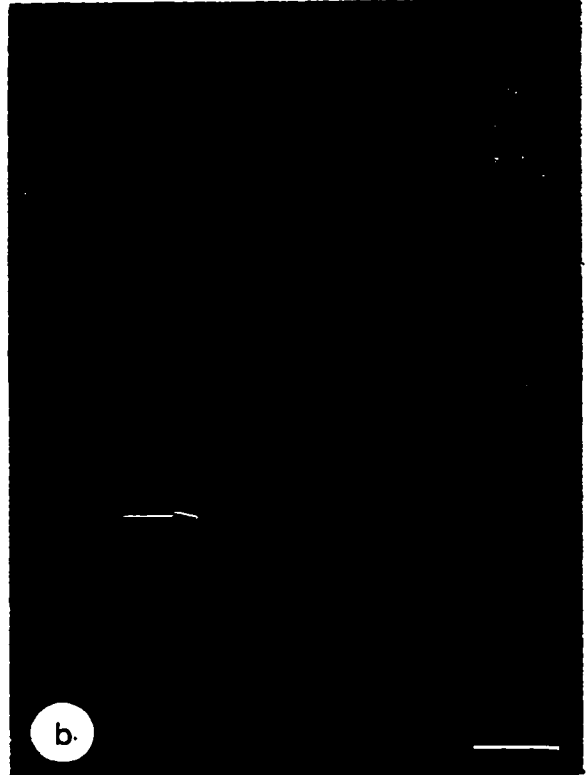


Fig. 13 The muscle bands and neurons of the strobila

- a) Muscle bands (arrow) extending from the scyphistoma region into the developing ephyrae; rhodamin phalloidin stain. Scale bar = 100 μ m**
- b) Swelling on muscle band (arrow) in the upper region of the strobila. Scale bar = 20 μ m**
- c) FMRFamide-positive neurons in the developing ephyrae. Scale bar = 10 μ m**
- d) MAb Aa-labelled fibre (arrowhead) in developing ephyra. Arrow points towards the scyphistoma part of the strobila. Scale bar = 20 μ m.**
- e) MAb Aa-positive neuron in ephyra about to be released. Scale bar = 5 μ m.**



amorphous substance (Fig. 14c) or with small granules of varying electron density (Fig. 14d). Nematocysts (Fig. 14e) are interspersed with the ectodermal cells and are particularly common in the tentacles. In the apical regions of the cells, septate desmosomes are frequently encountered (Fig. 14f). Some cells contain large as well as smaller, spherical to oval inclusions in close proximity to the nucleus (Fig. 15a). Small, dense-cored as well as clear vesicles are characteristically present in neuronal tissue (Fig. 15b). Muscle processes in the bases of the ectodermal cells insert into the mesogloea (Fig. 15c), and profiles of neurites that are found next to the muscle frequently contain small (100-140 nm) dense-cored vesicles (Fig. 15d). In endodermal cells of the body column, the ER surrounding the nucleus can form extensive cisternae (Fig. 15e), and the cytoplasm can contain numerous small, electron-dense granules (Fig. 15f).

The muscle cells extend from the tentacle onto the oral disc and have the appearance of smooth muscle (Fig. 16a). Neuromuscular synapses were not found, but neuro-neuronal synapses were (Fig. 16b), characterized by the presence of clear spherical synaptic vesicles (70-100 nm). In addition to the small dense-cored vesicles that are encountered relatively frequently in neuronal profiles, some fibres contain large dense-cored vesicles (exceeding 200 nm in diameter) (Fig. 16c). Both types can be found in close proximity to the membranes, but an actual release of vesicle contents has not been observed, although at times, electron dense material could be seen along the membrane (Fig. 16. d). When labelled with gold-conjugated anti-FMRFamide, some of the small dense-cored vesicles contained clusters of gold particles (Fig. 16 e, f).

Fig. 14 Electron micrographs of cells of the scyphistoma

a) Overview of cross section of ecto- (EC) and endodermal (EN) cells.

Scale bar = 10 μ m

b) Cross section of tentacle. EC: ectoderm; EN: endoderm; N: nematocyst.

Scale bar = 5 μ m

c) Ectodermal cell with inclusions filled with amorphous substance (arrowhead).

M: muscle process; arrow points to mesogloea; scale bar = 1 μ m

d) Ectodermal cell containing spherical inclusions filled with granules

(arrowhead). Scale bar = 0.5 μ m

e) Nematocyst in ectoderm; n: nucleus; scale bar = 1 μ m

f) Septate desmosome (arrowheads) in apical region of ectodermal cell.

Scale bar = 0.25 μ m

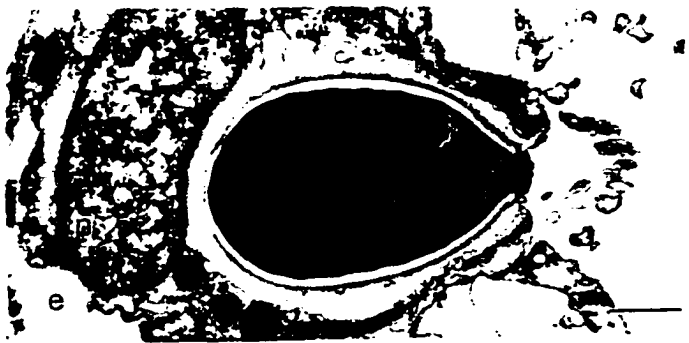
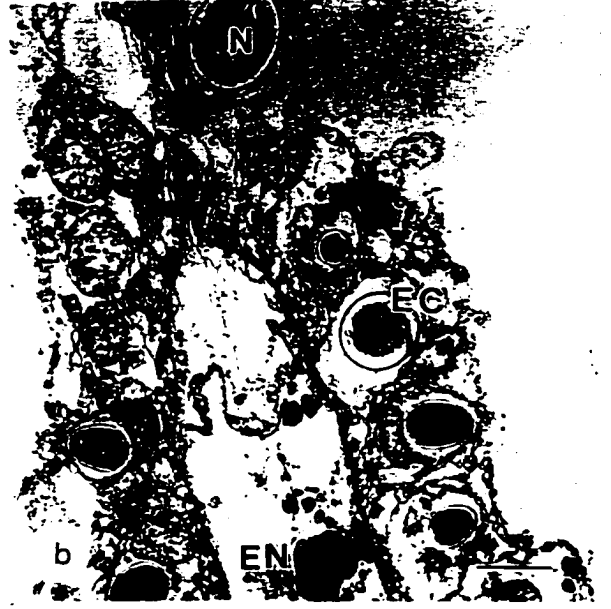
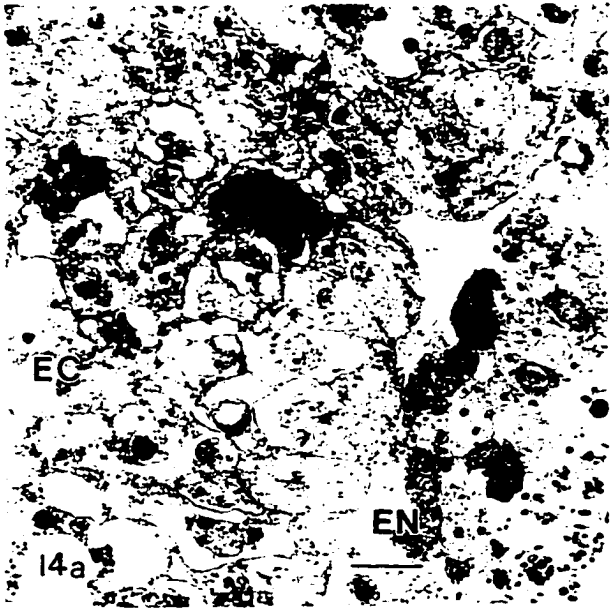


Fig. 15 Electron micrographs of ecto-and endodermal cells in the scyphistoma

- a) Contents of ectodermal cell include granules (curved arrow) as well as numerous inclusions, some of which are filled with an amorphous substance (arrow), others contain material of varying electron density (arrowhead); m: mitochondrion; n: nucleus; scale bar = $1\mu\text{m}$
- b) Part of neuron with dense-cored vesicles (dcv); scale bar = $0.5\mu\text{m}$
- c) Process of smooth muscle (M); N: nematocyst; n: nucleus; scale bar = $1\mu\text{m}$
- d) Profiles of smooth muscle (M) processes, accompanied by neurite with dense-cored vesicle (dcv); arrowhead points to mesogloea; scale bar = $0.5\mu\text{m}$
- e) ER of endodermal cell forming large cisternae (arrow) surrounding the nucleus (n); scale bar = $1\mu\text{m}$
- f) Endodermal cells with numerous small electron-dense granules (arrow). N: developing nematocyst; scale bar = $1\mu\text{m}$

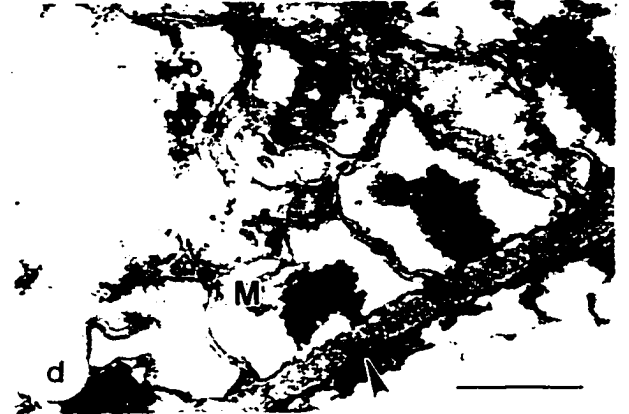
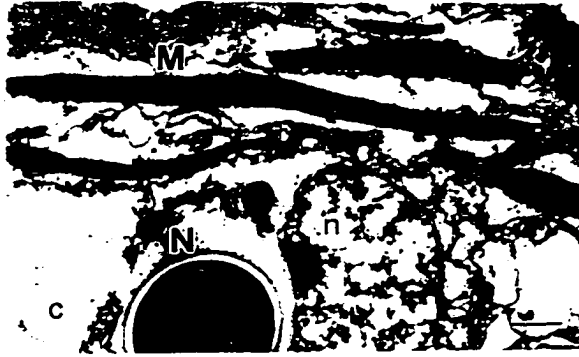
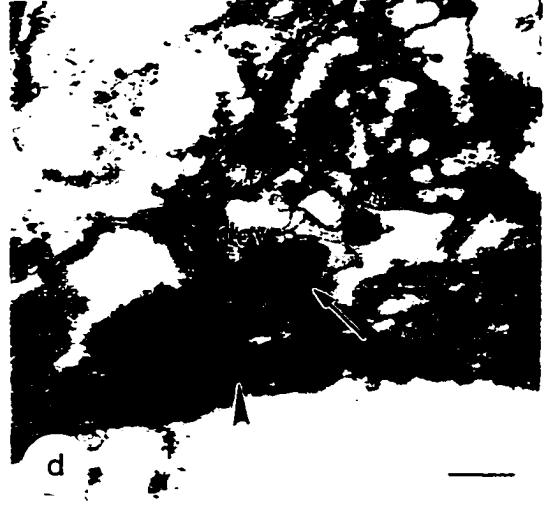
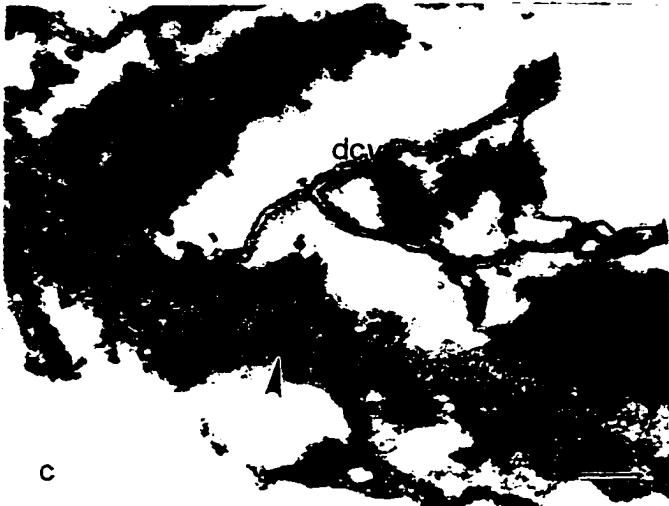
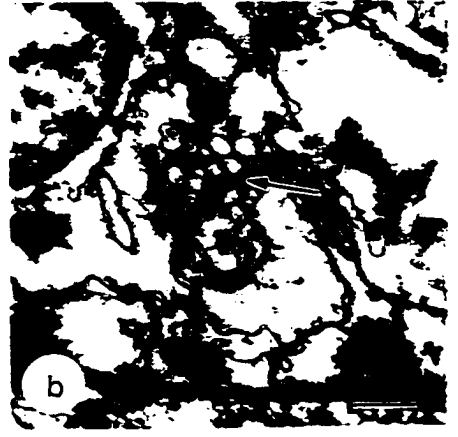


Fig. 16 Muscle and neuronal cells in the scyphistoma

- a) Two muscle processes (M) accompanied by neurite (arrow); scale bar = $1\mu\text{m}$
- b) Neuro-neuronal synapse (arrow) characterized by presence of clear synaptic vesicles; scale bar = $0.25\mu\text{m}$
- c), d) Two types of dense-cored vesicles (dcv) found in neurites; in d) presence of electron-dense material close to opposing membrane (arrow); arrowhead points to mesogloea; scale bar for c) and d) = $0.25\mu\text{m}$
- e), f) Labelling with anti-FMRamide antibodies and gold-conjugated secondary antibody; clusters of gold particles (arrows) can be seen on some dense-cored vesicles; arrowheads point to mesogloea. Size of gold particles in e) = 5 nm and in f) = 15 nm; scale bar for e) and f) = $0.5\mu\text{m}$



Discussion

The most prominent feature of the nervous system of the scyphistoma (at low magnification, at least) is the system of neurites extending from the oral disc onto the four muscle cords. The close association of FMRFamide-positive neurites with these muscle cords suggests that the neurons are involved in the innervation of the muscle, although how they accomplish this is not certain. In Anthozoa, according to McFarlane *et al.* (1989), RFamide, which induces contractions, acts directly on muscle cells. On the other hand, Antho-RFamide, which increases the frequency of spontaneous contractions, may be a neurotransmitter or neuromodulator, but where or how it acts is unknown. McFarlane *et al.* (1989) assume that it is delivered by directed synapses, but do not rule out a more widespread release, *e.g.* even into the coelenteron. Anctil (1989) suggests that in the Cnidaria, an evolutionary transition is beginning to take place from a paracrine to a transmitter role for neuroactive substances. However, based on their studies in bullfrog ganglia, Jan *et al.* (1983) propose a hypothetical scheme in which neuropeptides in general act as hormones, having the ability to diffuse for many micrometers from their release sites. The latter can be varicosities, but even when released from nerve terminals, in which case the peptides can be co-localized with other transmitters in synaptic vesicles, they can act upon cells that are not in synaptic contact with the peptidergic terminals. The scheme allows for the suggestion by Brumwell and Martin (1996) that FMRFamide is released at non-synaptic sites along the length of the neurites. Whether FMRFamide is also released from synaptic vesicles at synapses, however, remains to be seen. Moreover, I did not find any neuromuscular synapses, and their exact nature is still to be determined.

The presence of the neurites on the muscle cords had previously been mentioned (Chia *et al.*, 1984), however, the existence of single or pairs of fibres in the ectoderm of the body column had not been known. This is not surprising, however, since they

are few in number (one to two fibres per quadrant) and could easily be missed at the electron microscope level. Their function in the body column is not clear. They are not associated with muscle tissue, and presumably do not receive multiple input from other fibres, since they are not intimately associated with the nerve net. However, since one cannot assume that anti-FMRFamide labels all of the neurons in the scyphistoma, they may make contact with a number of other neurons. On the other hand, they could be involved with setting the threshold for nematocyst discharge.

The appearance of the FMRFamide-positive neurons on the oral disc and tentacles is that of a typical nerve net and was not unexpected. However, *Chia et al.* (1982) report the presence of bundles of up to 100 processes, and those were not encountered.

There seems to be a general consensus that the nervous system of the scyphistoma of *Aurelia* consists only of one type of conducting system (*Chia et al.*, 1982; Chapman, 1965). Gwilliam (1960) also reported only one system for the stauromedusa *Halichystus*. Compared to other coelenterates, they therefore have a much simpler organization of their nervous system. In the Anthozoa, three separate conducting systems and different types of sensory cells have been reported (*McFarlane et al.*, 1989), as well as the presence of a number of possible neurotransmitters such as serotonin and noradrenaline (*Anctil*, 1989).

The finding that the muscle bands of the scyphistoma do not participate in the formation of the ephyrae was more unexpected, since, during stolon formation, the cords bifurcate to provide the muscle for the developing polyp, and a similar process seemed at least plausible for the development of the ephyrae, which possess large amounts of smooth muscle. Perhaps the muscle cords provide part of an anchor for the ephyrae, which are free to separate from the strobila once the muscle has atrophied.

The system of FMRFamide-positive fibres in the developing ephyrae is similar to that in the released animal, but how it is established is unclear. Clearly new neurons

must be developing, since one source of neurons, the tentacles, is not involved in strobilation, and the number of neurons on the (one) oral disc are not enough to account for all the neurons in the stack of ephyrae. The appearance of only a few scattered mAb Aa-positive neurons in the developing ephyrae is in keeping with the pattern of labelling in the developing (released) ephyrae.

As to the results at the electron microscope level, one question which was not resolved pertained to the presence of striated muscle in the scyphistoma. Chapman (1965) observed a "hint" of irregular striations, but Chia *et al.* (1982) report striated muscle at the base of the tentacle. Sections of this area (Fig. 15c) could possibly be interpreted as possessing very slight striations, but they were not very convincing. While Chia *et al.* (1982) point out that variations may exist in the same species of *Aurelia*, they examined specimens from Washington State, and it seems unlikely that they are much different from local specimens. Certainly the ones I have examined myself for mAb Aa label did not show any difference from local animals.

The Free-Swimming Jellyfish: Ephyra, Juvenile and Adult

Introduction

The ephyra has most often been mentioned as a developmental stage of the life cycle of Scyphozoa. Outside this context, it has received little attention in the literature, and studies on the nature of its nervous system are even sparser.

Haeckel (1881), examining the life cycle of *Aurelia*, *Pelagia*, and *Chrysaora* from the scyphistoma to the ephyra, did not describe any nervous structures other than the tentaculocysts of the developing and free-swimming ephyra. Claus (1883) erroneously proposed that the tentaculocysts of the developing ephyra originated from

the tentacle stumps of the scyphistoma, and that the endoderm of the latter provided the beginnings of the statocysts. According to Claus, a pair of ectodermal swellings, which he referred to as "ganglia", developed at the base of the tentaculocyst of *Aurelia*. The latter is also an incorrect observation, since the only paired structures at the base of the tentaculocyst are protrusions from the gastric canal, and the only nervous structure which could be considered a 'ganglion', *i.e.* the accumulation of neurons in the tentaculocyst, is not paired. Goette (1887) disagreed with Claus (1883) as to the origin of the ephyral tentaculocysts, but instead believed that they originated from the subumbrellar surface, and that the statocysts only formed in the ephyra. He found that, before the release of the ephyra, ectodermal and endodermal tissue elements connecting it to the remainder of the scyphistoma shrank and atrophied. Goette's observations correspond to the processes in the developing ephyrae of *Aurelia aurita* from the Pacific Northwest and Japan.

Some short notes on ephyral aberrations and food-capture are worth mentioning since they shed some light on the general biology of this developmental stage, whereas the most comprehensive paper on the neurobiology of the ephyra was published by Horridge (1956a). Low (1921) investigated variations in the ephyrae of *Aurelia aurita*. He found that about 80 % of the strobilae he examined produced both normal and abnormal ephyrae, and that approximately 30 % of the total number of ephyrae produced showed abnormalities, the majority of which occurred in the number of arms. For example, one strobila produced ephyrae whose arms ranged in number from four to twelve. Since the arms in four-armed specimens consisted of the interradial arms only, the latter appeared to be more constant than the perradial arms. Other abnormalities included imperfect arms, *i.e.* missing lappets, missing or 'twin' tentaculocysts, and extra or missing gastric filaments. Gemmill (1921) examined ephyrae of *Aurelia aurita* regarding food-capture and ciliation. When food particles such as ciliates touched the arms of an ephyra, nematocysts discharged into the prey,

whose movements were further hampered by mucous produced by the ephyra, and by the action of the nematocyst toxin. Either all arms or just the arm concerned stopped beating, and the arm then bent towards the manubrium, which in turn bent toward the arm carrying the food. The lips stretched open widely in the same direction, and the arm was wiped over the mouth. [This manner of food uptake is very similar to that of the scyphistoma, where the tentacles laden with food bend towards the mouth, which opens widely in the direction of the food.] The detached ciliates were then slowly carried into the gastric cavity by, according to Gemmill (1921), "a central inhalant current which is compensatory to exhalant currents produced by ciliary action in the floor of the mouth angle grooves". Most of the ciliates were already dead when ingested, and accumulated in the gastric cavity with "surprising rapidity". [Not all of the captured ciliates that are still alive manage to escape but are further exposed to the densely packed nematocysts of the gastric filaments.] Food particles that were captured on the surface of an ephyra were swept away by ciliary currents. Gemmill (1921) observed that on the exumbrellar surface and the subumbrellar surfaces of the arms and the outer disc, the currents flowed centrifugally outwards, towards the lappets. On the remainder of the subumbrellar disc, the currents flowed centripetally inwards, directing the prey towards the mouth. Internally, the direction of the currents was centrifugally outwards on the roof of the gastric cavity and of all the gastric canals, and centripetally inwards on the floor of the gastric cavities and of all the gastric canals. The currents also ran from base to tip of each gastric filament, but ciliation was ill-defined in the manubrial canal, except for the floor of the radial grooves, where it was weakly exhalant. While young ephyrae (1-5 days old) were successfully feeding on ciliates, Gemmill (1921) did not find any crustacean remains in over 2000 animals.

Horridge (1956a) investigated the neuronal control of ephyral behaviour. He examined two distinct kinds of ephyral response, the feeding reaction and rhythmical swimming, in an attempt to determine the corresponding elements of the nervous

system. His observations regarding food-uptake were similar to Gemmill's (1921), although he reported that his animals, which were 3-5 mm in diameter, were able to catch copepods. [Although smaller animals are most likely newly released, size is not necessarily a good indicator of age. In my own cultures, I have observed animals of the same age that ranged in size from 3-8 mm. The size of the newly released ephyra seems to depend on the size of the scyphistoma, which in turn seems to be influenced by the feeding conditions and the available space. Smaller animals often have difficulty capturing (3-day-old) *Artemia* sp. nauplii, even when fed vigorously, and can stay the same size for several weeks, until they most often die.] Horridge (1956a) referred to the independent action and contraction of each arm, resulting in the capture of food particles in rapid succession by different arms, as the "feeding reaction", and the sustained contraction of the whole animal, resulting from stronger stimulation, as the "spasm". During the spasm, which may last for several minutes, all the arms fold tightly over the subumbrellar surface, and the animal falls through the water. The "beat", on the other hand, refers to the rhythmical and symmetrical swimming movements and is quite distinct.

As mentioned in the Historical Overview, Horridge (1956a) was able to visualize elements of the giant fibre system and the diffuse nerve net, using reduced methylene blue in his histological preparations. The giant fibre system consists of a network of highly oriented bipolar nerve cells that are mostly associated with the radial and circular muscle, except for some neurons that connect with the tentaculocysts or cut across the corners at the base of an arm. The somata are 6-10 μ m long, and the fibres less than 1 μ m in diameter. Horridge also observed some [presumed] synapses, most of which were of the single contact type, but many of which made multiple contacts. In the latter case, fibres ran alongside each other for as much as 30 μ m. Although he did not find any specialized nerve endings on the muscle, Horridge did see sharp twists of

axons round a muscle fibre. In his most favourable preparations, he counted no more than 25 neurons in a $300\mu\text{m}^2$ area of radial muscle.

In contrast to the giant fibre system, the cells of the diffuse nerve net spread over the whole of the epithelium and consist of bipolar sensory cells, bi- and (mostly) multipolar cells covering the exumbrellar and subumbrellar surfaces, and bipolar cells with long processes oriented along the arms and round the manubrium. The fibres of the diffuse nerve net are thin and have many twists compared to the broader and straighter giant fibres. They, too, make connections with the tentaculocysts. The bipolar cells with long processes that are found on the arm can stretch almost the entire length from the lappets to the disc. They contact the peripheral sensory cells and the multipolar cells of the remainder of the diffuse nerve net. The bipolar cells along the manubrium connect with a network of fibres which spreads over the tip of the manubrium and extends on the inside towards the gastric cavity. Other fine fibres from multipolar cells are found in the epithelium directly overlying the gastric filaments. [Horridge did not indicate whether he thought that these cells were endodermal.] Cells of the diffuse nerve net also appeared in specimens with rudiments of marginal tentacles. Some of the latter were bipolar cells extending one fibre towards the mouth while the other branched profusely within the bud, and others were sensory cells.

In the tentaculocysts, Horridge (1956a) distinguished between four kinds of cells, which he labelled "a"- "d". He noted that the relationship between cells and fibres showed a marked bilateral symmetry. Type "a" cells are a group of bipolar cells at each side of the base of the tentaculocyst. One of their processes, which are of the giant fibre type, contact the giant fibre system on the radial muscle, whereas the other ascends into the tentaculocyst and branches among the cells of type "b". The latter are bi- and multipolar cells in the apex of the tentaculocyst. Their short processes, whose branches form a dense network of fibres, ramify to the tip of but never leave the tentaculocyst. Type "c" cells are represented by a pair of bipolar cells near the base of

the ganglion, one on each side. Each has a short process that branches among the cells of type "a", and a long process that branches among the corresponding cells on the other side. Finally, the cells of type "d" are found in the side of the tentaculocyst, about midway to the tip. They are bipolar cells whose short process branches among the cells of type "b" and whose long process contacts fibres of the diffuse nerve net outside of the tentaculocyst. Horridge thought that the type "b"- "d" cells were ectodermal because of their orientation and position in the epithelium and because he occasionally observed a short process extending to the surface. He did not find any endodermal nerve cells in the tentaculocysts, although he did not know the origin of the type "a" cells.

In addition to the determination of the histological structures, Horridge conducted a number of experiments both on whole and on parts of animals. In specimens with all but one arm removed, he observed that the remaining arm and the disc continued to beat, and that the radial and the circular muscles contracted together at each beat. A slight touch on the lappets elicited a feeding response, and a delicate mechanical stimulus on the circular muscle sometimes resulted in a local, maintained contraction, whereas a strong touch to any part of the remaining arm or the disc caused a spasm. Horridge considered four of his experiments to be decisive in his investigation of the existence of two distinct nerve nets. Incisions from the base of each arm towards the manubrium that were sufficient to cut through the circular muscle and the giant fibre net associated with it resulted in the arms beating independently from each other. The spasm, however, occurred as usual. Secondly, in an isolated arm where a cut on the side of the tentaculocyst severed the fibres from the tentaculocyst to the radial muscle band, only the strip of muscles still in communication with the tentaculocyst continued to beat, but the feeding response or the spasm could involve the whole of the arm. Thirdly, in a specimen with two tentaculocysts on one arm, a cut between these caused the two sides of the isolated arm to beat

independently, but the coordination of the spasm remained intact. Finally, ephyrae that are about to be released from the strobila beat independently of each other, but a stimulus at the base of the strobila produces successive spasms of each ephyra.

Horridge was satisfied that his anatomical and experimental results were in accordance. He concluded that the giant fibres coordinated the beat, and the diffuse nerve net the feeding reaction and the spasm. The neurons in the tentaculocysts controlled the spontaneous rhythm and allowed for the interaction of the two nerve nets. Horridge did not find any evidence that the two nets interacted where they overlie the muscle. He favoured the concept of a double motor innervation by both of the two nets over the possibility that two kinds of muscle fibre existed, since his histological results did not indicate any evidence for the latter. [As mentioned before, however, there *is* smooth muscle in the ephyra.] Since both the feeding reaction and the spasm were attributed to the diffuse nerve net, it had to be arranged in sectors corresponding to the arms. The spasm, then, was a feeding reaction that had spread to all the sectors and to the circular muscle. The presence of cells of the diffuse nerve net in tentacle rudiments and their connections to cells of the same net on the manubrium led Horridge to believe that the tentacle wave of the adult was also brought about by the diffuse nerve net. He also pointed out that both in the ephyra and the adult, the diffuse nerve net was more widespread than the giant fibres. In the ephyra, the spasm persisted while the beat did not in the cutting experiments.

Horridge (1956a) was the first to thoroughly investigate the existence of two nerve nets in the ephyra. He histologically identified their components and experimentally demonstrated their involvement in different ephyral behaviours. However, a number of questions remain open. For example, did Horridge determine the full extent of the ephyral nervous system, or are there other components and perhaps even other nerve nets present? Which are the transmitters involved and what are their functions? Can components of the nervous system be directly associated with

different behaviours, *i.e.* is it possible to histologically identify them individually? In general, what are the ultrastructural qualities of the ephyral tissue? In addressing these questions, I first reexamined Horridge's (1956a) methylene blue results, and further investigated other means of labelling and staining in order to reveal more identifying characteristics for different neurons or subsets of neurons. As in the scyphistoma, anti-FMRFamide antibodies labelled components of the diffuse nerve net. The immunofluorescence revealed the presence of a net of FMRFamide-positive neurons in the sub- and exumbrellar surfaces, and FMRFamide-positive components in the endoderm. I further generated monoclonal antibodies to a subset of neurons of *Aurelia* (the first and only ones produced so far) which recognized individual neurons that appeared scattered over all surfaces. The labelled neurons included components (bi- and tripolars) of the giant fibre net in the subumbrella, and bi- and multipolar neurons in the exumbrella, but did not form a nerve net in the ephyra.

In addition, I investigated the effect of various stimuli, such as *Artemia* filtrate and FMRFamide, on the swimming behaviour of the ephyrae. In order to minimize the effect on ephyral swimming of physical contact between free-swimming nauplii with ephyrae, I used Millipore filtered *Artemia* concentrate. I included a control with distilled water (added amounts were equivalent to added FMRFamide), since the FMRFamide was originally resuspended in distilled water. Because Henschel (1935), who investigated the "chemical senses" in adult scyphomedusae, found that all the amino acids and protein compounds he tested elicited a feeding response, *i.e.* they were transported and taken up into the mouth, I examined the effects of FM[D-R]Famide (in order to determine whether FM[L-R]Famide was recognized as a food substance), and glycine.

In a similar but separate set of experiments, I identified the neurons that were active in the presence of different stimuli with the activity-dependent dye rhodamine B. Rhodamine B is a fluorescent dye which, like other rhodamine compounds, can be used

to stain living cells. Unlike Rhodamine 123, however, which is positively charged and has been used to stain mitochondria in presynaptic nerve terminals (Yoshikami and Okun, 1984), Rhodamine B is uncharged and possibly enters cells via synaptic vesicle recycling, demonstrated at the frog neuromuscular junction (Betz and Bewick, 1992), and so it can be used as an indicator of activity. I quantified the results and tried to determine the involvement of the diffuse and the giant fibre nerve nets.

As far as the ultrastructure of the ephyra is concerned, I examined the ephyral tissue at the electron microscope level and identified FMRFamide-positive neurons using gold as a label.

The ephyra represents an important stage in the life cycle of *Aurelia*, since it is the beginning of the free-swimming existence, which the animals adopt until their deaths. Although the planulae are free-swimming, too, that stage is much shorter, and swimming is brought about by ciliary movement. Also, while ephyral feeding movements and the net of FMRFamide-positive neurons are reminiscent of those in the scyphistoma, in the ephyra, striated swimming muscles and the neurons innervating them are developed for the first time, as are sensory organs in the form of the tentaculocysts, which possess a statocyst and act as pacemakers. Both ephyral neuronal and muscular elements are building blocks that are expanded during the development of the ephyra until the adult structures are accomplished. It is of interest to determine the characteristics of those elements, not only because they are of importance in the ephyra, but also because their subsequent contribution during the development to and in the adult can be monitored.

As the ephyra develops into the adult jellyfish, the space between the arms is filled in so that the lappets-to-arms-ratio increases while the length of the arms decreases. Near the margin, tentacles form as does the circular canal of the gastric cavity, and the manubrium develops into frilled oral arms. Gastric canals, some of which branch, divide the gastric cavity and radiate towards the margin. The bell

becomes scalloped and the gonads form. Throughout development, the number of gastric filaments and the amount of mesogloea continually increase.

Spangenberg (1965) cultured *Aurelia* from the Gulf of Mexico in artificial sea water and divided the ephyral development into nine stages. I have not adhered to Spangenberg's stages for a number of reasons. She herself concedes that "it is not possible to correlate closely either size or age of the organism with the development of any specific morphological structure." Further, there are some inconsistencies in both the sequence of developmental events and some morphological features between her animals and those of the Pacific west coast. For example, she states that new features develop in a consistent chronological sequence and that the circular canal appears before the marginal tentacles, whereas I have seen tentacle rudiments in specimens where the circular canal still developed. Also, she reports that the adradial gastric canals are branched in an earlier stage whereas they are unbranched later on.

[According to Kozloff (1987) *Aurelia aurita* of the Pacific Northwest have unbranched adradial but branched inter- and perradial canals.] Although there are some general tendencies in the sequence of events, given that the ephyral development is very gradual and given that different features develop simultaneously but not necessarily in the same time frame, I find it difficult to divide it into (nine) stages that are consistent, both from animal to animal, and in the number and weight of changes, *i.e.* what occurrence (or combination) of feature(s) constitutes a new stage? The one characteristic feature of Spangenberg's stage VIII, for example, is only increased ruffling of the oral arms.

For the purposes of this study, 'ephyra' denotes any animal, regardless of size, with eight well developed arms, as well as a manubrium. In the juveniles, the space between the arms is filling in, the manubrium is beginning to develop into the oral arms, and tentacles develop. Also, the gastric filaments are increasing in numbers and the gastric canals are beginning to branch. This stage is often accompanied by a loss of

pigmentation. Any animal, regardless of size, in which the developmental processes of the juvenile were completed, was termed an adult, even if sexual maturity could only be determined by the presence of gonads.

I have followed the development of the nervous system from the ephyra into the adult, using anti-FMRFamide and the Aa monoclonal antibody.

Materials and Methods

The following are materials and methods not covered in Chapter 2, General Materials and Methods.

Methylene Blue Staining

The reduced methylene blue was prepared according to Unna (1916). Three drops of 24% hydrochloric acid were added to 100 ml of 0.5% methylene blue in distilled water. 5 ml of the mixture were heated in a test tube in a water bath of boiling water and 12% rongalite (BDH Chemicals Ltd., Poole, England) was added drop by drop (4-6 drops) until the solution just turned clear. It was allowed to cool, the test tubes were covered and let stand overnight (24-36 h). [This solution is good for 8-10 days.] Care was taken not to disturb the test tubes, as this tended to increase the rate of re-oxidization of the methylene blue. For the same reason, the solutions were not filtered, but rather, small amounts were removed with a Pasteur pipette. Eight drops were added to live ephyrae in 30 ml of sea water. After varying lengths of time, the ephyrae were removed to sea water, immediately mounted on a slide in a small amount of sea water and viewed on a Leitz Aristoplan microscope.

Immunofluorescence for Double-Labeling

For double labelling, ephyrae fixed in Zamboni's fixative (Zamboni, 1967) were incubated for 3 hours to overnight at room temperature in undiluted hybridoma supernatant to which anti-FMRFamide (1:100), 0.1% Triton X-100, 2% goat and 2% donkey serum and 0.3% sodium azide had been added. They were then washed three times in TRIS buffer, pH 7.0, for 15 minutes each and incubated for 2 hours to overnight in rhodamine-labelled goat anti-mouse IgG+M (H+L) plus fluorescein-labelled donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., Mississauga, ON) in TRIS buffer. The washing steps were repeated and the specimens mounted on slides in PBS-Glycerol (1:1) with 0.15% N-propyl-gallate added to retard fading, and 0.03% sodium azide. The preparations were examined by epifluorescent illumination of a Leitz Aristoplan microscope at either 546 nm (rhodamine excitation) or 485 nm (fluorescein excitation). Photographs were taken with ASA 400 film.

Rhodamine B Staining

A 1% stock solution of Rhodamine B in sea water was filtered with a 0.22 μ m filter, wrapped in aluminium foil and stored in the refrigerator. The final dilution of 0.01%-0.05% was made up just prior to use with sea water to which different treatments had been added. The treatments used to test activity-dependent uptake of rhodamine B were 10⁻⁶M and 10⁻⁷M FMRFamide and FM[D-R]Famide, 0.13M and 0.19M magnesium chloride, 200 μ l *Artemia* sp. filtrate and approximately 2 ml of whole *Artemia* (100-200 animals) per 10 ml final solution. The MgCl₂ was prepared by adding appropriate amounts of a 0.11M solution of MgCl₂ in distilled water to sea water. When available, 10 ephyrae of apparent identical developmental stage were used for each treatment. The animals stayed in the solutions for 2 hours and were then

washed in several changes of sea water for 1-2 hours, during which time the containers were covered with aluminium foil. The specimens were then mounted on slides in a small amount of sea water and viewed with a rhodamine filter on a Leitz Aristoplan microscope. Photographs were taken with a Kodak ASA 3200 film.

Effects of Treatments (FMRFamide, Artemia, and Glycine) on the Swimming Beat

Ephyrae were exposed to 10^{-6} and 10^{-7} M FMRFamide, 10^{-6} and 10^{-7} M FM[D-R]Famide, 200 μ l *Artemia* filtrate, and 10^{-6} M glycine in Millipore filtered seawater. The controls consisted of Millipore filtered seawater alone, and 10-100 μ l of distilled H₂O per 10 ml of seawater. Swimming beats were counted for 20 min.

Results

Gross Morphology of the Ephyra

The ephyra stage is generally less than 1 cm in diameter and characteristically has, radiating from the disc, eight arms that each bifurcate peripherally into two lappets (Fig. 17a). Each arm possesses a tentaculocyst, situated between the bases of the lappets, and two bands of radial muscle. A continuous band of circular muscle is found near the margin of the disc. The ephyra possesses only two types of nematocysts (Calder, 1977), which are especially prominent along the sides of the arms (Fig. 17b, c). The manubrium is located in the center of the subumbrellar surface. The uptake of sulphorhodamine 101 demonstrates the extension of the gastric cavity in the arms and into the tentaculocysts (Fig. 17d). Surrounding the area below the base of the manubrium are four groups of gastric filaments (evaginations of the floor of the gastric cavity). Both the radial and circular muscles contract during swimming, but,

particularly in animals with a small disc, most of the water displacement is due to the bending of the arms, which come together closely, (rather than radiating out from the disc), resulting in a dome-shaped appearance of the animal. During feeding, arms that have come in contact with prey move towards the mouth, the manubrium bends towards the arm and the mouth opens wide, and the food is released into the mouth. Ephyrae, particularly smaller specimens, are poor predators of 3 day old *Artemia*. In a small bowl with plenty of free-swimming *Artemia*, it can take up to 45 min. until a nauplius is successfully captured, even though the ephyra may have had numerous encounters with prey. The latter, however, is frequently successful in freeing itself, even when it appears to have caused the discharge of nematocysts. Abnormal ephyrae (Fig. 17e) frequently have missing or extra arms, deformed arms or extra tentaculocysts and lappets, and occasionally possess two manubria.

Light Microscopical Observations

The Muscles

The striated radial muscle bands of the arms extend from the striated circular muscle into the lappets (Fig. 18a, b). Smooth muscle is found between the radial muscle bands (Fig. 18c), in the tips of the lappets, on the disc radiating towards the manubrium, and on the manubrium (Fig. 18d,e). The latter has four bands of longitudinal muscle between which smooth muscle is arranged perpendicularly.

Methylene Blue Staining

Staining with methylene blue produced results that were comparable to those of Horridge (1956a), not only as far as the different types of stained cells are concerned,

but also in regard to his notes on methodology. Individual results varied, *e.g.* even in animals from the same 'batch' (animals that were exposed to the same preparation of methylene blue for the same length of time), the staining pattern varied in terms of types, numbers, and parts of cells that were stained. Herein lie both advantages and disadvantages of this method in that it is possible to follow the fibres of individual cells or have only cells of one of the nerve nets stained in some preparations, although not all of the cells stain at all times. In general, cells of the giant fibre system showed up first whereas cells of the diffuse nerve net were sometimes difficult to see and did not stain all at once. Also, somata stained more quickly than processes and eventually, the entire animal turned blue.

Cells of the giant fibre system were associated with the radial and circular muscle and extended all the way into the lappets (Fig. 19a, b). The somata reached lengths of $12\mu\text{m}$ and the fibres diameters of $1\mu\text{m}$. In the diffuse nerve net, varicosities of the processes were much more pronounced compared to the giant fibres (Fig. 19c). The cells, which compared in size to those of the giant fibre system (lengths of somata of up to $12\mu\text{m}$, fibre diameter of up to $0.8\mu\text{m}$), were also found associated with the muscle bands, but sensory cells and cells on the exumbrellar surface also stained. In the tentaculocysts, all four types of cells identified by Horridge (1956a) and fibres entering and leaving this structure were stained (Fig. 19d, e), although the type "c" cell was most elusive.

Anti-FMRFamide Label

Cells that were labelled with the anti-FMRFamide antibodies seemed to be part of the diffuse nerve net. In the region below the tentaculocyst, the net was less dense and individual cells and fibres could be seen most easily (Fig. 20a). As for the tentaculocysts themselves, cell bodies and fibres inside as well as fibres approaching the

Fig. 17 The ephyra of *Aurelia aurita*

a) Eight arms radiate from the central disc and bifurcate peripherally into two lappets (L). The tentaculocysts are located at the base of the bifurcation (arrow), and the mouth is located in the center of the disc (arrowhead). Scale bar = 0.5 mm.

b) Large nematocysts (arrowhead) are found along the sides of the arms. Scale bar = 20 μ m.

c) The two types of ephyral nematocysts (arrow and arrowhead). Scale bar = 5 μ m

d) Uptake of sulforhodamine shows extent of gastric cavity (arrow). Scale bar = 0.5 mm

e) Malformations in the ephyra can take the form of stunted arms (arrowhead) and partial fusion of arms (arrow). Scale bar = 1 mm.

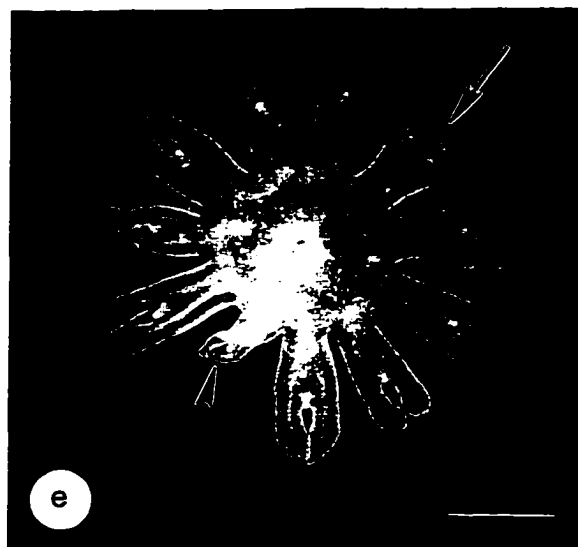
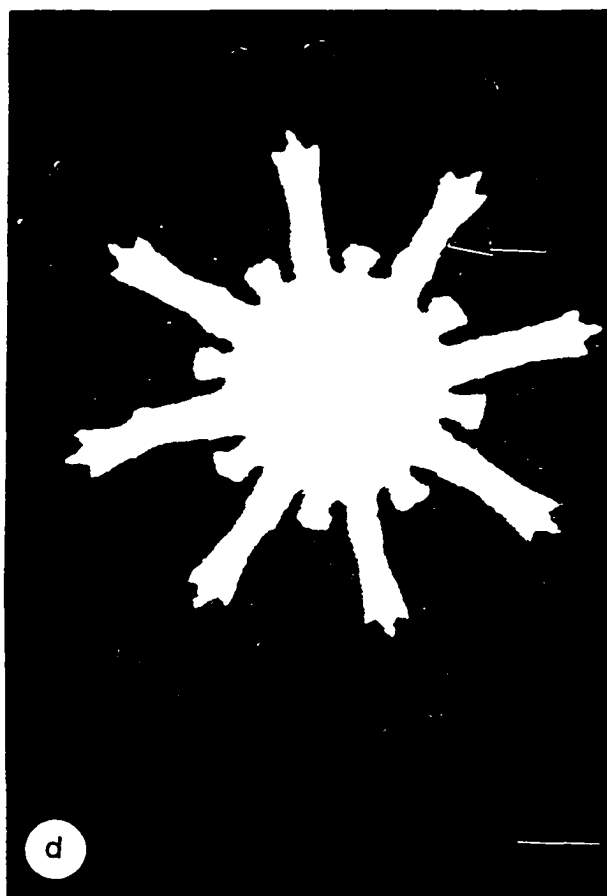
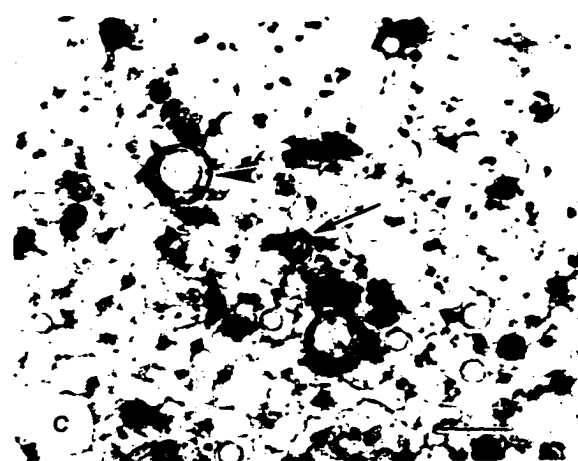
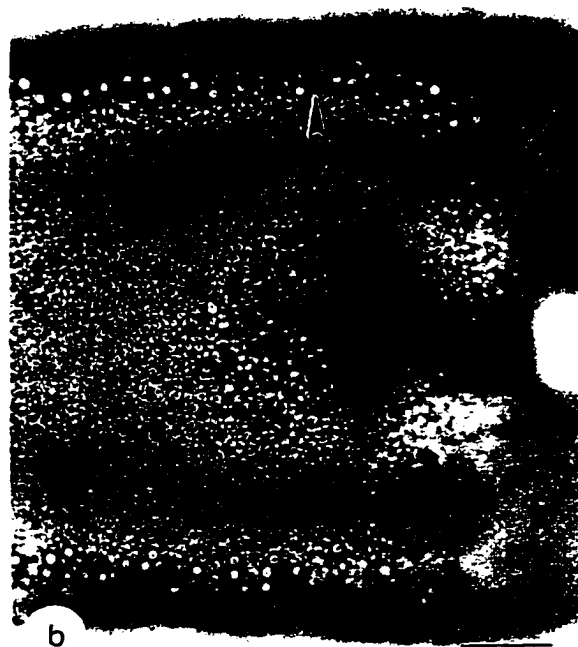
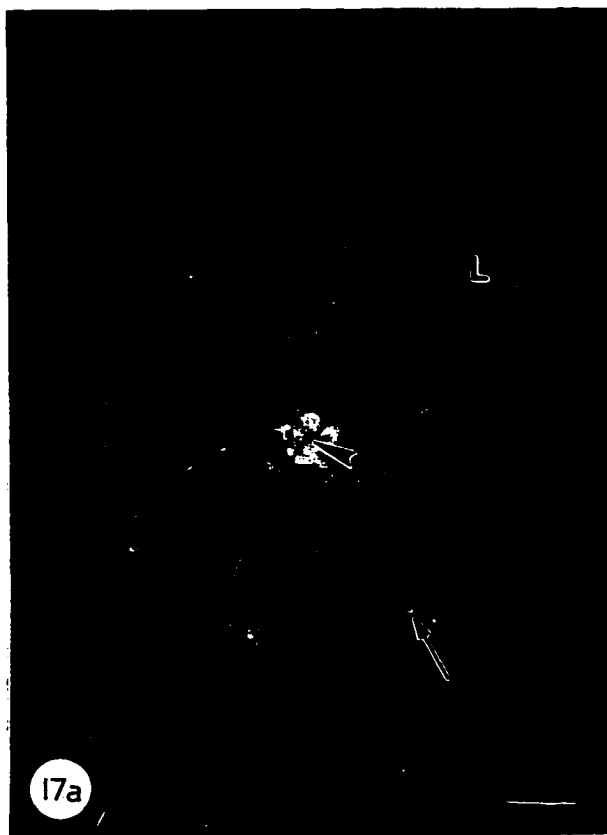


Fig. 18 The muscle systems of the ephyra

a) Arrangement of radial (RM) and circular (CM) muscles; arrowhead: tentaculocyst. Scale bar = 50 μ m. Inset: extension of muscle into the lappet.

Scale bar = 20 μ m

b) Circular muscle at higher magnification, showing striations.

Scale bar = 10 μ m

c) Smooth muscle (arrow) between radial muscle bands (RM).

Scale bar = 10 μ m

d) Band of smooth muscle on the manubrium (MA) and on the disc (D).

Scale bar = 10 μ m

a), c) and d) were stained with NBD-phalloidin, b) with rhodamine phalloidin.

e) Diagram of muscle arrangement; stippled: striated circular (CM) and radial (RM) muscle; lines: smooth muscles of manubrium (MA) and disc.

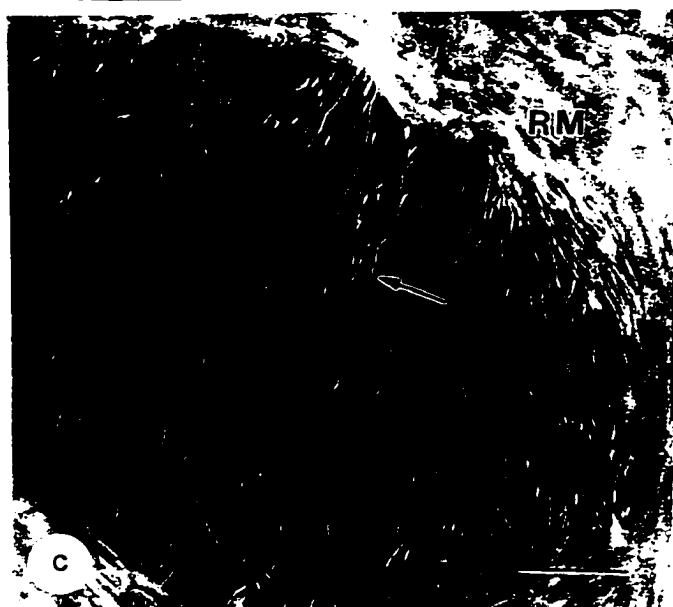
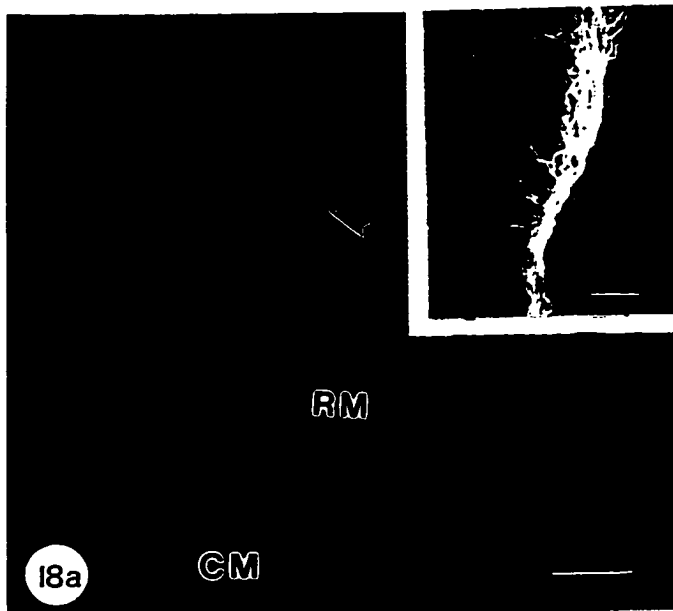


Fig. 19 Methylene blue staining of ephyral neurons

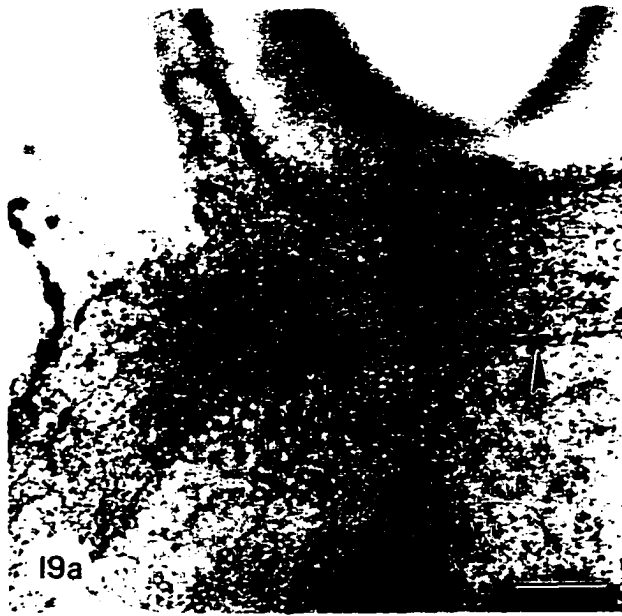
a) Cell bodies (arrowhead) and fibres of the giant fibre system on the circular muscle. Scale bar = 20 μm

b) Higher magnification of a). Cell body (arrowhead) on the circular muscle is extending a fibre toward the radial muscle. Scale bar = 10 μm .

c) Fibre (arrow) of the diffuse nerve net showing varicosities.
Scale bar = 10 μm .

d) Tentaculocyst with neurons of type "a", "b", and "d". Arrowhead: statocyst.
Scale bar = 10 μm .

e) Diagram of neurons in tentaculocyst. Inset: proposed wiring diagram after Horridge (1956a).



tentaculocyst were seen. The appearance of a nerve net was most closely achieved on the exumbrella, the sides of the arms, the lappets, and the margin and center of the disc (Fig. 20b). Here, the cells and fibres covered the surfaces with many twists and turns of the cell processes and many apparent contacts between the fibres. On the manubrium, the fibres were more parallel to one another and were arranged in the direction of the muscle bands. Accumulations of cell bodies were found in developing tentacles (Fig. 20c). On the radial and circular muscles, the fibres tended to be straighter and more parallel to each other, but were not as stout as the giant fibres, and often appeared intertwined for about $50\mu\text{m}$ and sometimes even for $100\mu\text{m}$ (Fig. 20d).

In the gastric filaments (Fig. 20e), some of the neuronal cell bodies were located at the base of the endodermal cells, while others were sensory cells. The fibres, which extended the entire lengths of the filaments, made contact with FMRamide-positive cells on the inside of the manubrium.

The whole of the exumbrellar surface was covered by labelled cells and processes (Fig. 21a). The latter cells were mostly multipolar, but there were also few bi- and tripolar cells. The nerve net persists in the developing juvenile/young adult, although it appeared to be less dense (Fig. 21b). On the exumbrella, the number of cell bodies decreased from $16/100\mu\text{m}^2$ in the ephyra to about $5/100\mu\text{m}^2$ in older stages, while on the developing manubrium, the network of fibres associated with the longitudinal muscle bands became more dense and the fibres increased in number from between 15-18 fibres/ $100\mu\text{m}$ to 34 fibres/ $100\mu\text{m}$.

The pattern of the label was found to be similar in ephyrae of *Pelagia* (Fig 21c) and *Chrysaora* (Fig. 21d, e), although on the subumbrellar disc of the latter, several fibres appeared to be twisted around one another in the shape of an arch, several of which encircled the manubrium.

Fig. 20 Neurons labelled with anti-FMRamide

a) Neurons and fibres below the tentaculocyst. Arrow: labelled cells in the tentaculocyst. Scale bar = $10\mu\text{m}$

b) Nerve net on the disc (D) and the manubrium (MA). Scale bar = $20\mu\text{m}$

c) Accumulation of cell bodies in developing tentacle (arrowhead).

Scale bar = $10\mu\text{m}$

d) Fibres appear to be twisted around one another in the area between arrows.

Scale bar = $10\mu\text{m}$

e) Neurons in gastric filaments (GF). Scale bar = $20\mu\text{m}$

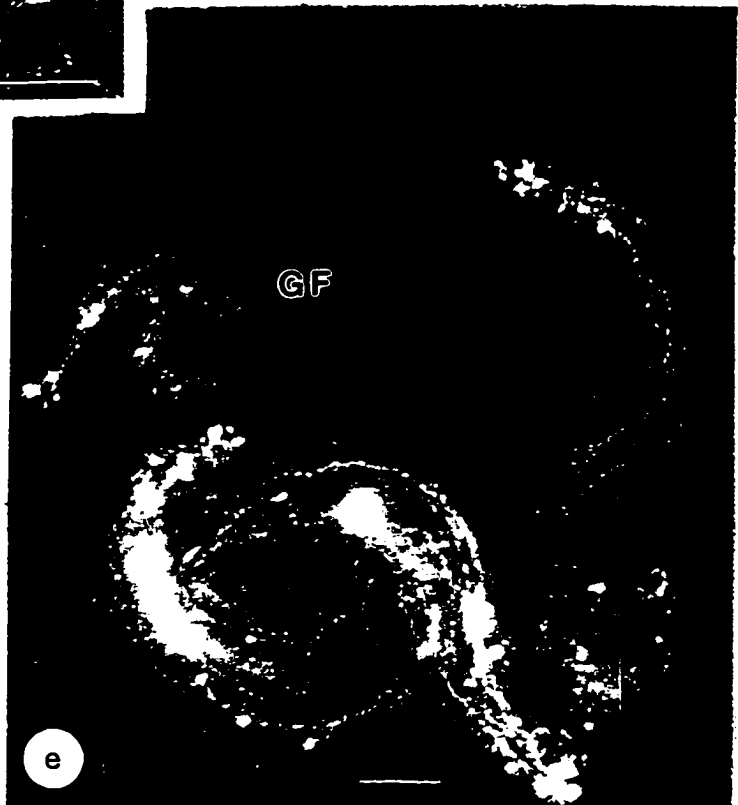
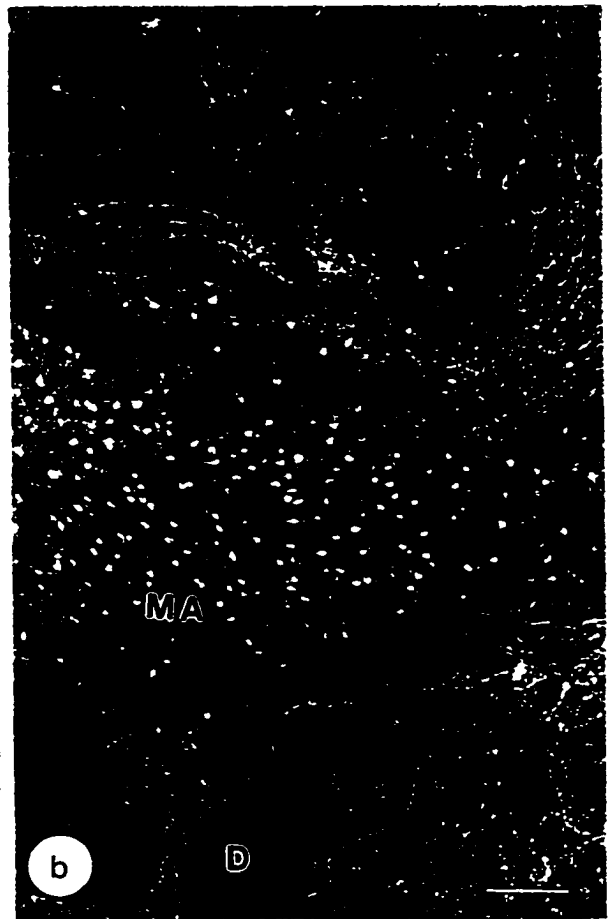
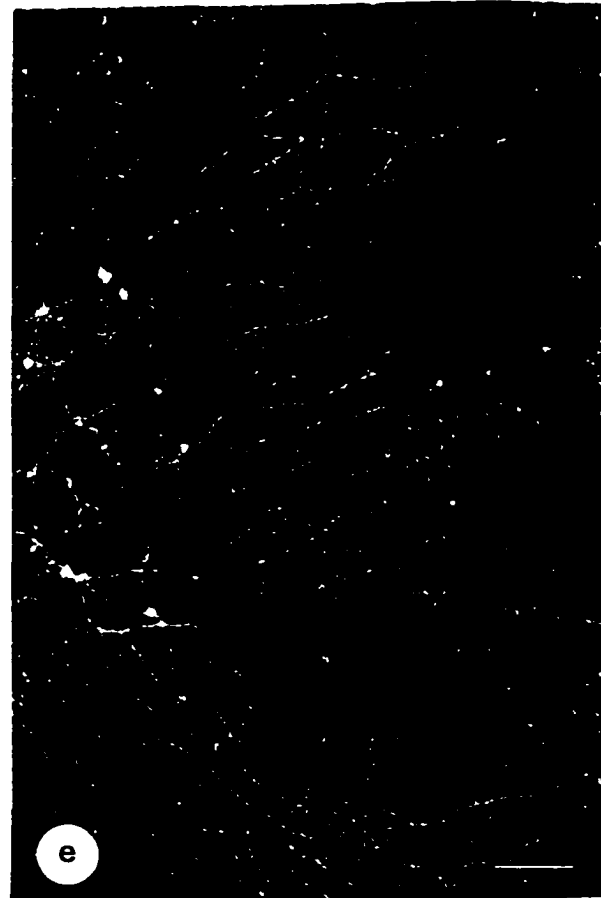
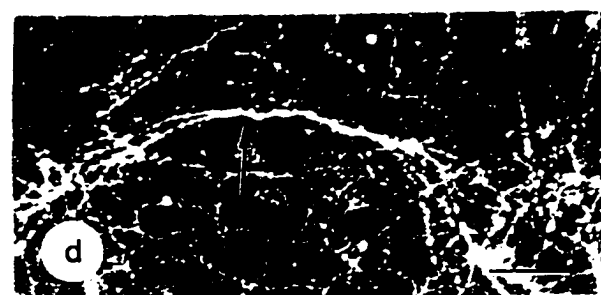
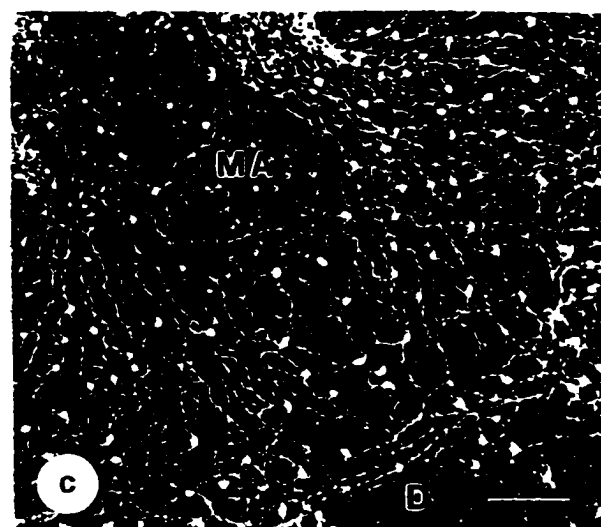
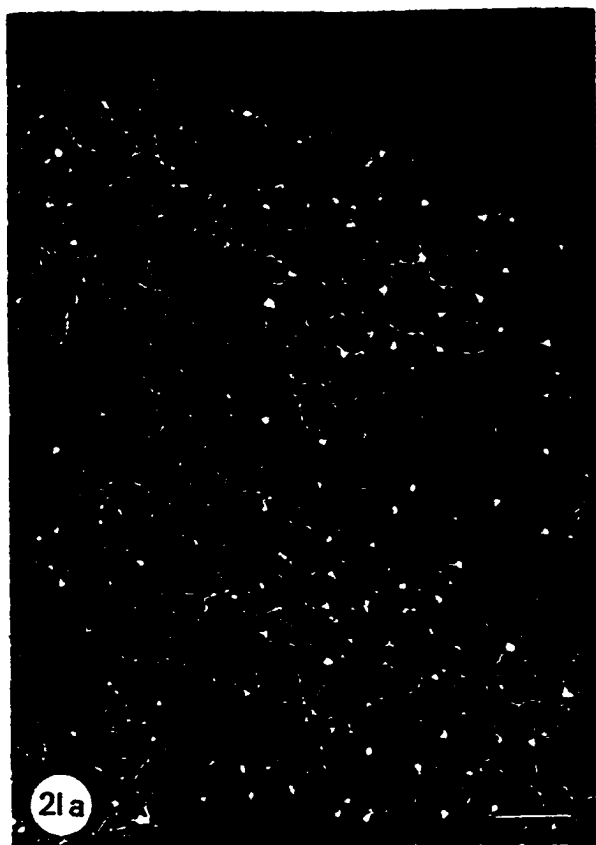


Fig. 21 Anti-FMRFamide-labelled neurons in the ephyra

- a) Neurons on the exumbrellar surface. Scale bar = 20 μ m
- b) Exumbrellar nerve net in the juvenile. Between arrows: extended area with reduced density of fibres. Scale bar = 20 μ m
- c) Disc (D) and part of manubrium (MA) of *Pelagia*. Scale bar = 20 μ m.
- d, e) Nerve net in *Chrysaora*; in d) fibres are intertwined (arrow); e) Subumbrellar disc. Scale bar = 20 μ m



Monoclonal Antibody Aa Label

Few cells were labelled with mAb Aa in the newly released ephyrae (as few as 10-20 cells in the entire animal), but the number of labelled cells increased as the animals developed (*e.g.* in animals that were still in the 8-arm stage but whose size had approximately doubled, more than 50 positive cells were found on the subumbrella surface alone). Different types of cells were labelled, and labelled cells were found in the ectoderm of the exumbrellar and subumbrellar surfaces, as well as in the endoderm. In the earlier stages, single neurons were labelled that were without any contact to one another. On the radial and circular muscle bi- and tripolar neurons resembling cells of the giant fibre system were labelled (Fig. 22a, b), and were associated with the muscle bands either along the sides or the middle of the bands. Other neurons extended one process along the muscle whereas the other was draped around it or double-backed. Cells on the radial muscle also sent fibres towards the periphery of the arm. Other, multipolar neurons had one fibre on the muscle while the other branched towards the middle of the arm or contacted one muscle band while extending one process towards the other muscle band. As development proceeded, more and more cells were labelled (Fig. 22c through e) until the fibres, which were oriented predominantly parallel to the muscle, seemed to form a net.

In the developing tentacle (Fig. 22f), fibres and, not uncommonly, a pair of cell bodies located at the base of the tentacle were labelled. Increased labelling was observed in the tentacle of the adult (Fig. 22g).

The same pattern of increased labelling in developmentally older stages was also found on the subumbrellar disc. Here, individual or pairs of mostly multipolar neurons were found in the ephyra (Fig. 23a). As the animals developed, fibre length as well as branching patterns increased until, again, the appearance of a nerve net was observed (Fig. 23b-d).

Fig. 22 MAb Aa label on striated muscle and in the tentacle

- a) Bipolar and b) tripolar cells on circular muscle. Scale bar = $10\mu\text{m}$
- c) through e) Increase in number of labelled neurons with progressing development. Scale bar in c) = $10\mu\text{m}$, in d) and e) = $20\mu\text{m}$
- f) Label in developing tentacle. Arrowheads: pair of labelled cells at base of tentacle. Scale bar = $10\mu\text{m}$
- g) Fibres labelled in tentacle of adult. Scale bar = $10\mu\text{m}$

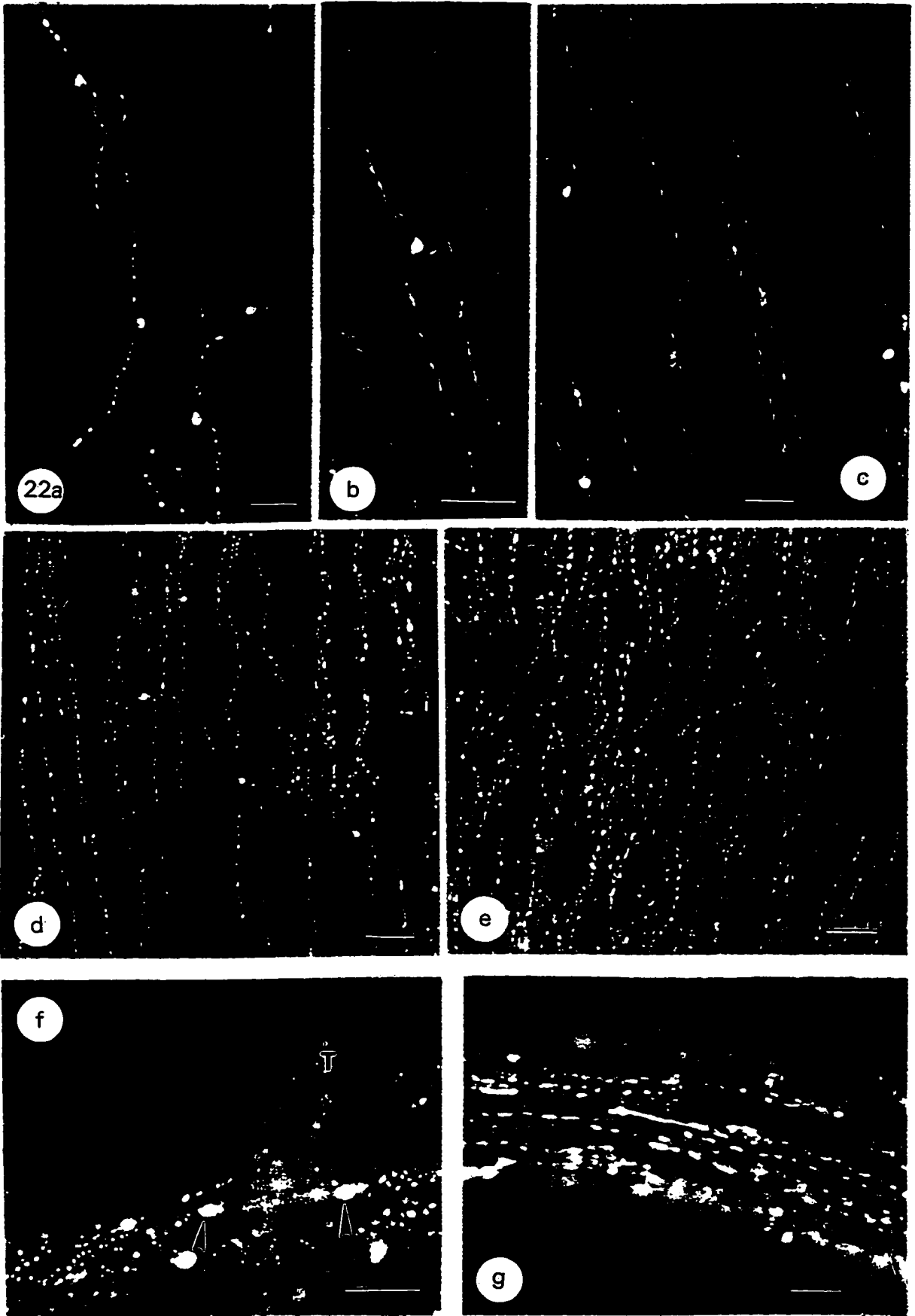
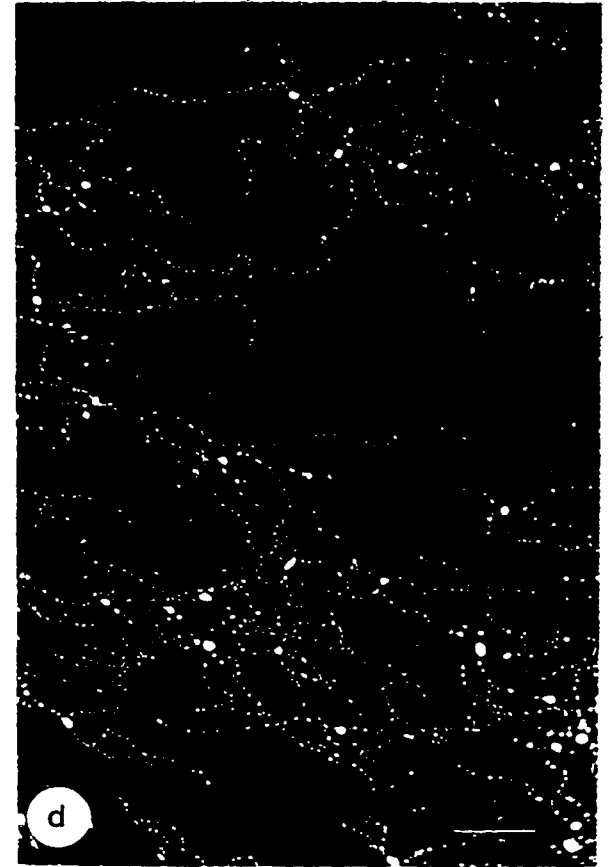


Fig. 23 MAb Aa label on the subumbrellar disc

- a) Two neurons in the ephyra; arrows point to cell bodies.
 - b) Three neurons in older stage than a), showing increase in branching pattern; arrows point to cell bodies.
 - c) and d) Further increase in labelling and appearance of nerve net.
- Scale bar for a)-c): = $10\mu\text{m}$; for d) = $20\mu\text{m}$



Tri- but mostly bipolar cells extended their processes along the manubrium (Fig. 24a). They were scattered over the surface in the ephyra, but as the manubrium increased in size, more cells were labelled and were oriented parallel to the fibres of the muscle cells in the bands and between the bands (Fig. 24b). The arrangement was similar to that of the FMRFamide-positive neurons of the manubrium (Fig. 24c). As the oral arms developed, more cells were labelled and appeared to radiate from the muscle band to the periphery (Fig. 24d, e).

The same increase in labelling in older animals was observed on the exumbrellar surface. Here, only a few tri- and multipolar cells were labelled in the ephyra (Fig. 24f). Labelled neurons were also seen extending their fibres from the exumbrellar to the subumbrellar surface, and from the outside of the manubrium to its inside surface (Fig. 24g).

Several types of cells were labelled in the tentaculocyst. Some could be classified according to Horridge (1956a) (Fig. 25a), but others could not be identified (Fig. 25b, c). Several cells in the vicinity of the tentaculocyst sent processes in its direction (Fig. 25d), some of which appeared to make contact.

Labelled cells in the endoderm were scattered over the endodermal surface of the ephyra (Fig. 26a), whereas in older stages, the fibres increased in length and grew towards one another, until they appeared to make contact (Fig. 26b, c)

Rhodamine B Staining

Rhodamine B consistently stained both sensory cells and neurons in the ephyrae (Fig. 26d, e; Fig. 27a). In the cell bodies, the nuclei did not stain, and stained fibres had a beaded appearance. Unfortunately, however, the degree of photobleaching was high, and it proved difficult to capture the stained neurons photographically.

Fig. 24 MAb Aa label on the manubrium and exumbrella

a) Bi- and tripolar neurons are labelled on the manubrium of the ephyra.

Scale bar = $10\mu\text{m}$

b) Labelled neurons in older stages are oriented along the muscle cells of the bands (arrows) and in between the bands (arrowheads).

c) Comparison to orientation of FMRFamide-positive neurons of the manubrium; arrows and arrowheads as in b). Scale bar for b) and c) = $10\mu\text{m}$

d), e) Increase of labelled neurons and appearance of nerve net on oral arms

Scale bar for d) and e) = $10\mu\text{m}$

f) Tripolar neurons in the exumbrella

g) Neurons on outside surface of manubrium (arrow) extend processes to its inside.

Scale bar for f) and g) = $20\mu\text{m}$

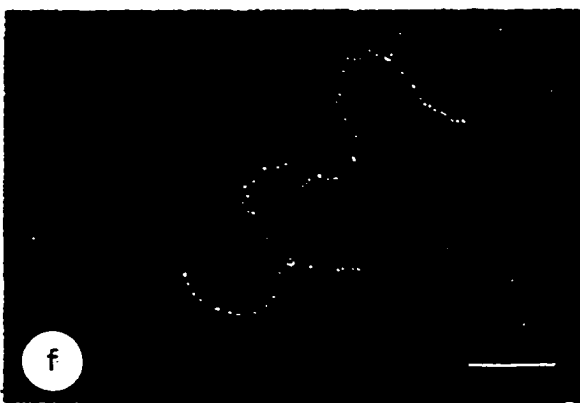
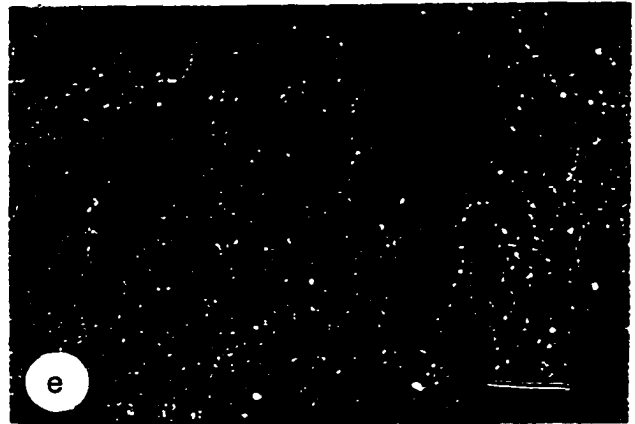
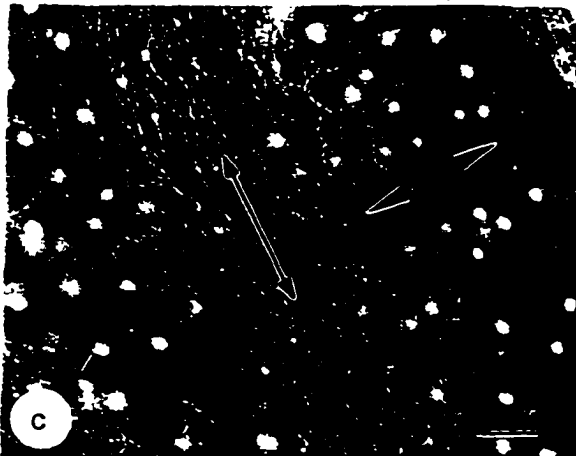
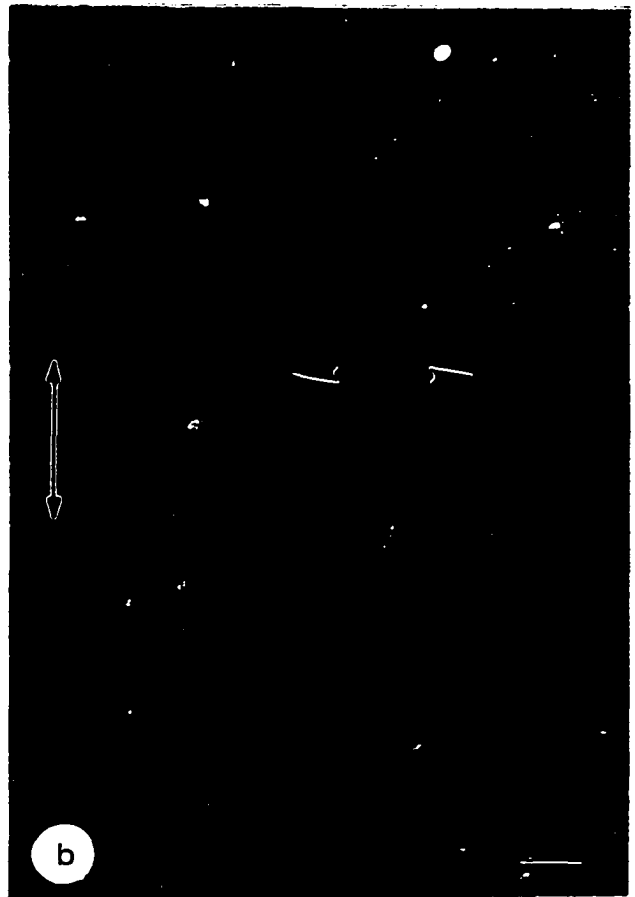
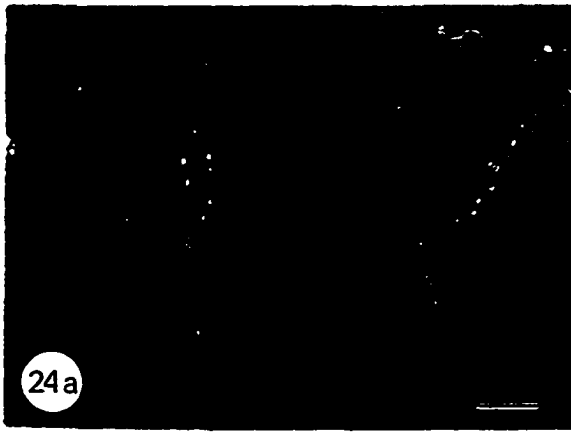


Fig. 25 MAb Aa label in the tentaculocyst

a) Neuron that could be of the "d" type. Other neurons are located on the lappet (L) and close to the tentaculocyst.

b) Neuron of unidentified type;

Scale bar for a) and b) = $10\mu\text{m}$

c) Diagram of distribution of labelled neurons labelled in the tentaculocyst

d) Tripolar neuron extending one process to the radial muscle, one towards the tentaculocyst, and one towards the mid region of the arm.

Scale bar = $10\mu\text{m}$

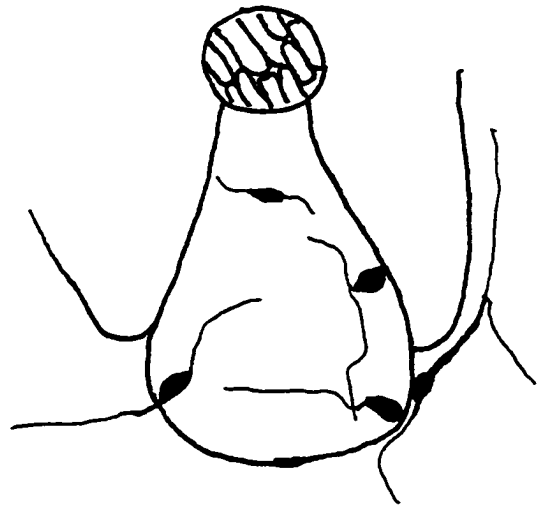
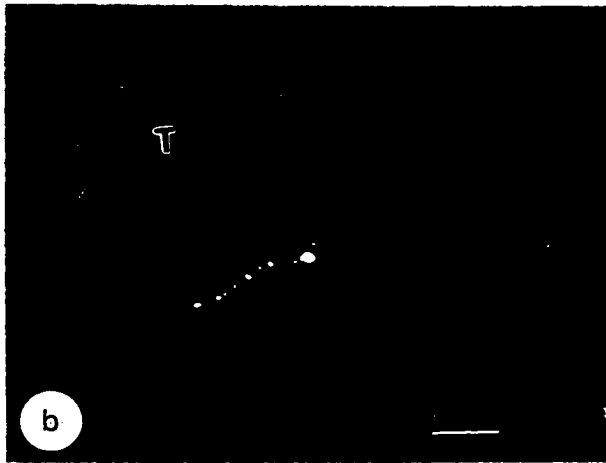
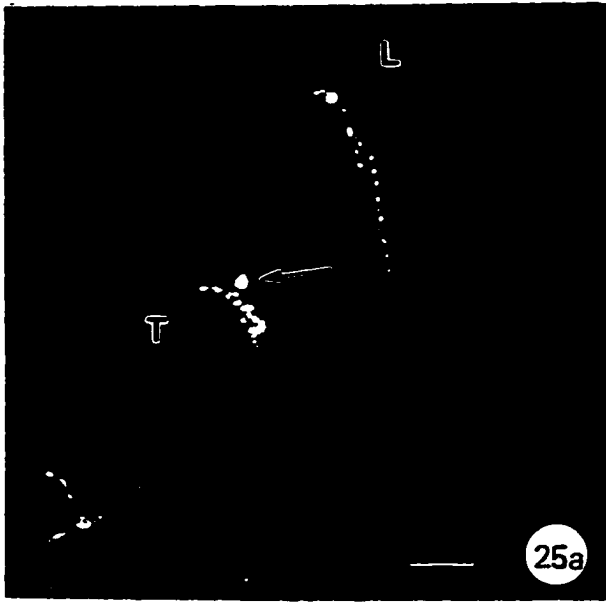
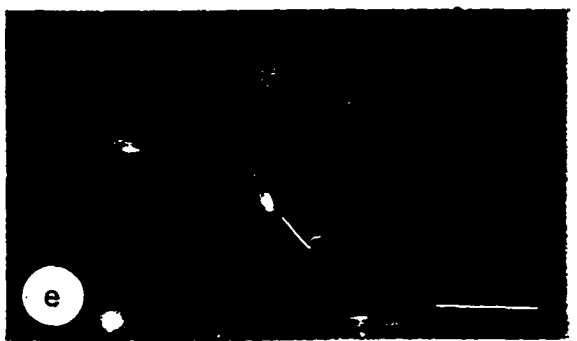
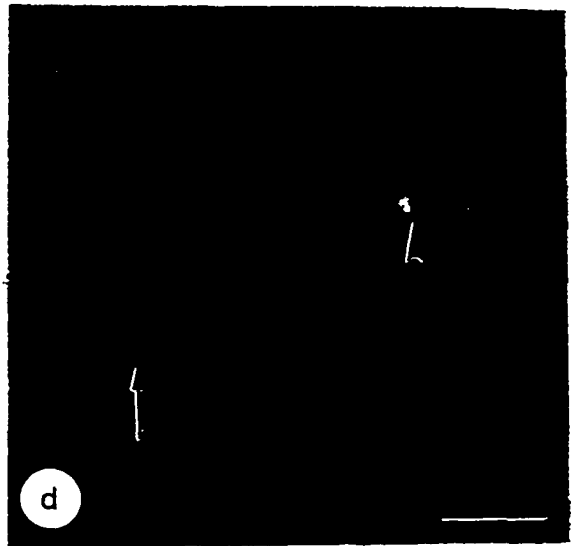
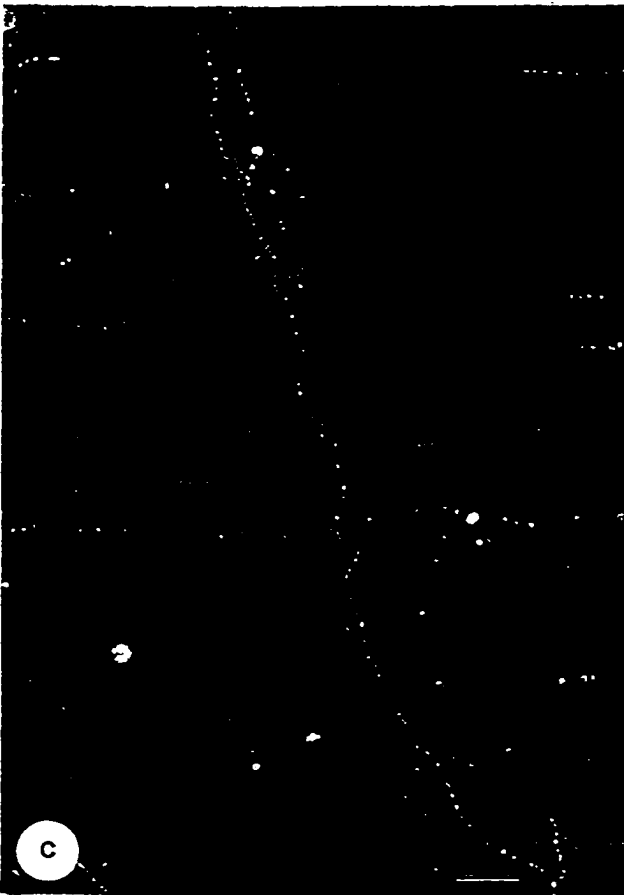
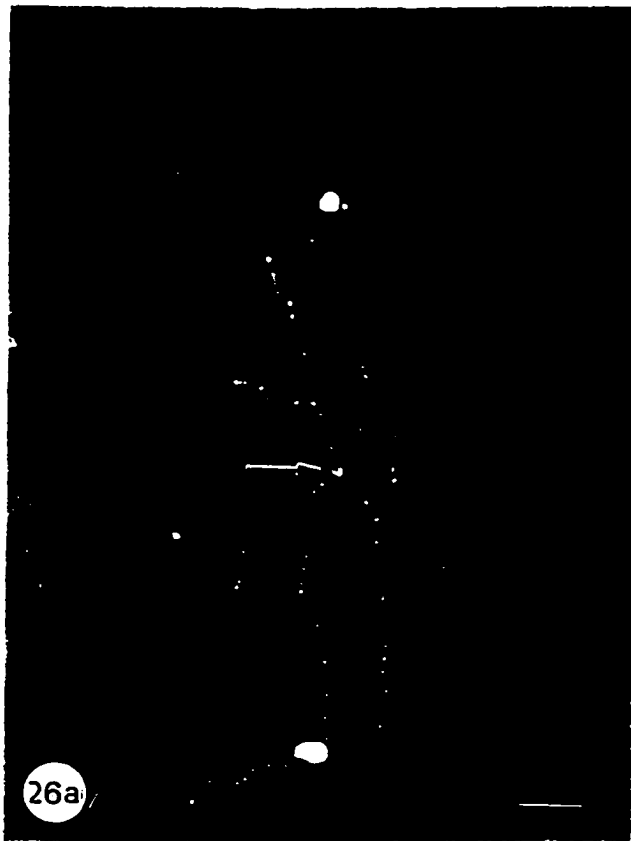


Fig. 26 MAb Aa label in the endoderm, and neurons stained with Rhodamine B

- a) Endodermal neuron in the ephyra. The arrow points towards the cell body.
- b) and c) Endodermal neurons labelled with mAb Aa in later stages, showing extension of fibres. Scale bar for a) - c) = $10\mu\text{m}$
- d) Neuron and e) sensory cell labelled with Rhodamine B. Arrowheads point to cell body, while arrow points to fibre. Scale bar for both = $10\mu\text{m}$



Double-labelling with anti-FMRFamide and mAb Aa

Double-labelling revealed no co-labelling in the ephyrae, although both cell types can be observed in close proximity (Fig. 27b, c). In the ephyra, there is no common orientation of the two types of fibre, while in older stages, the fibres appear to run parallel to one another. Comparison of the two labels in the ephyra shows the more uniform, nerve-net-like distribution of FMRFamide-positive neurons and the scattered distribution of neurons labelled with mAb Aa (Fig. 28)

Electron Microscopical Observations

The shape and dimensions of the ectodermal cells differ depending on whether they are found on the exumbrellar or subumbrellar surface. On the exumbrella, the cells are more box-shaped and frequently contain larger vacuoles (Fig. 29a). The cells are ciliated, the membranes are fairly straight, and the nuclei are found at the base of the cells. The cytoplasm contains various inclusions of different size and electron density. Some of the latter are round and filled with an amorphous, electron dense substance, while others are shaped more irregularly and their contents are less electron dense. Nematocysts are common on the exumbrella. Occasionally, amoebocytes are seen in the mesogloea (Fig. 29b). They are small, undifferentiated cells which contain ER and free ribosomes, mitochondria and inclusions with an electron-dense content. Ectodermal cells of the subumbrellar surface also contain vacuoles, but the latter are not consistently as big as the ones in exumbrellar cells. The nucleus is located centrally or towards the base. Among the cell contents are inclusions filled with an amorphous substance or with more structured components, and vesicles with varying contents are frequently found just below the apical pole of the cells (Fig. 29c). Interspersed among the ectodermal cells are nematocytes and -blasts. Most prominent in the subumbrellar

Fig. 27 Rhodamine B stain and double-labelling of neurons in the ephyra

a) Rhodamine B stain; arrowhead: cell body, arrow: fibre with beaded appearance.

b) and c) Double-labelling with anti-FMRFamide and mAb Aa. FMRFamide-positive neurons are green, those labelled with mAb Aa are red.

Scale bar for a)-c) = 10 μ m.

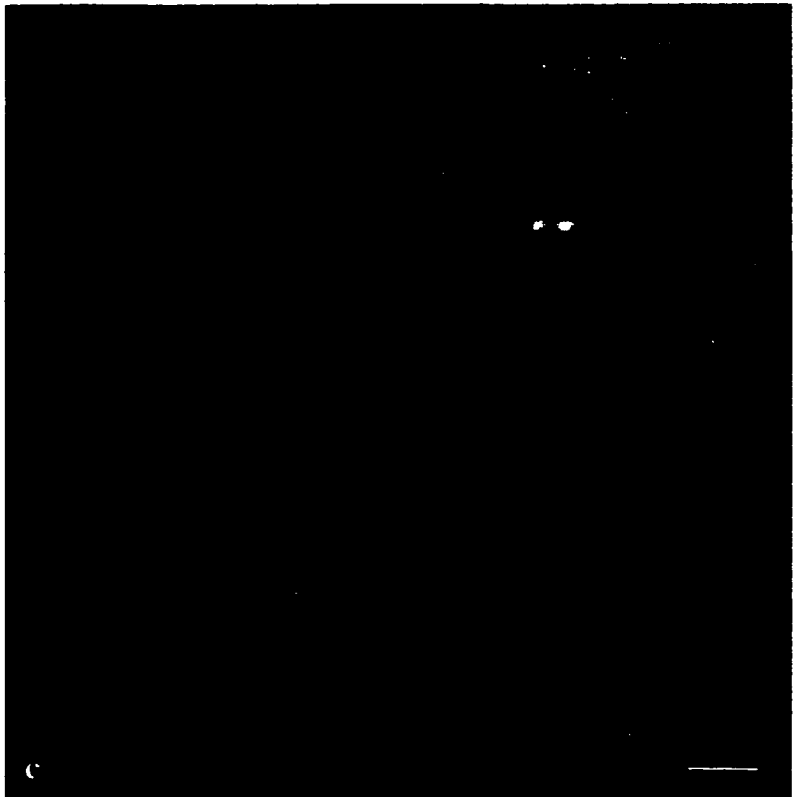
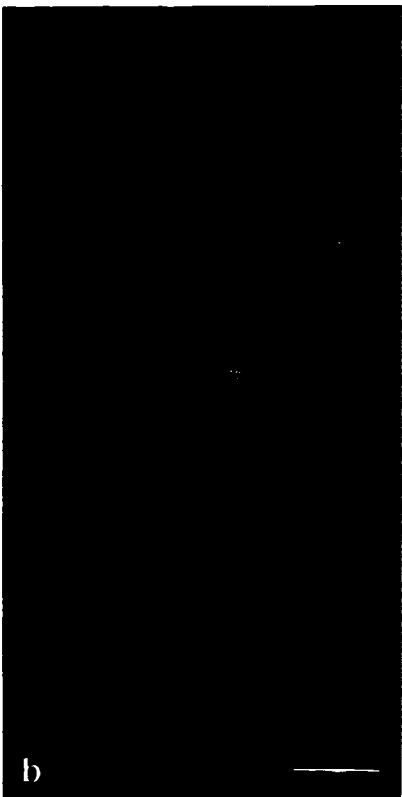


Fig. 28 Diagrammatic representation of distribution of neurons

Neurons labelled with anti-FMRFamide are on left and those labelled with mAb Aa are on right. The upper half of the drawing represents the subumbrellar, and the lower half the exumbrellar surface. Neurons drawn on the manubrium (MA) and the extension of the gastric canal (GC) are labelled with monoclonal antibody, while the ones in the tentacle bud (TB) are FMRFamide-positive. CM and RM: circular and radial muscle, respectively; T: tentaculocyst, and D: disc.

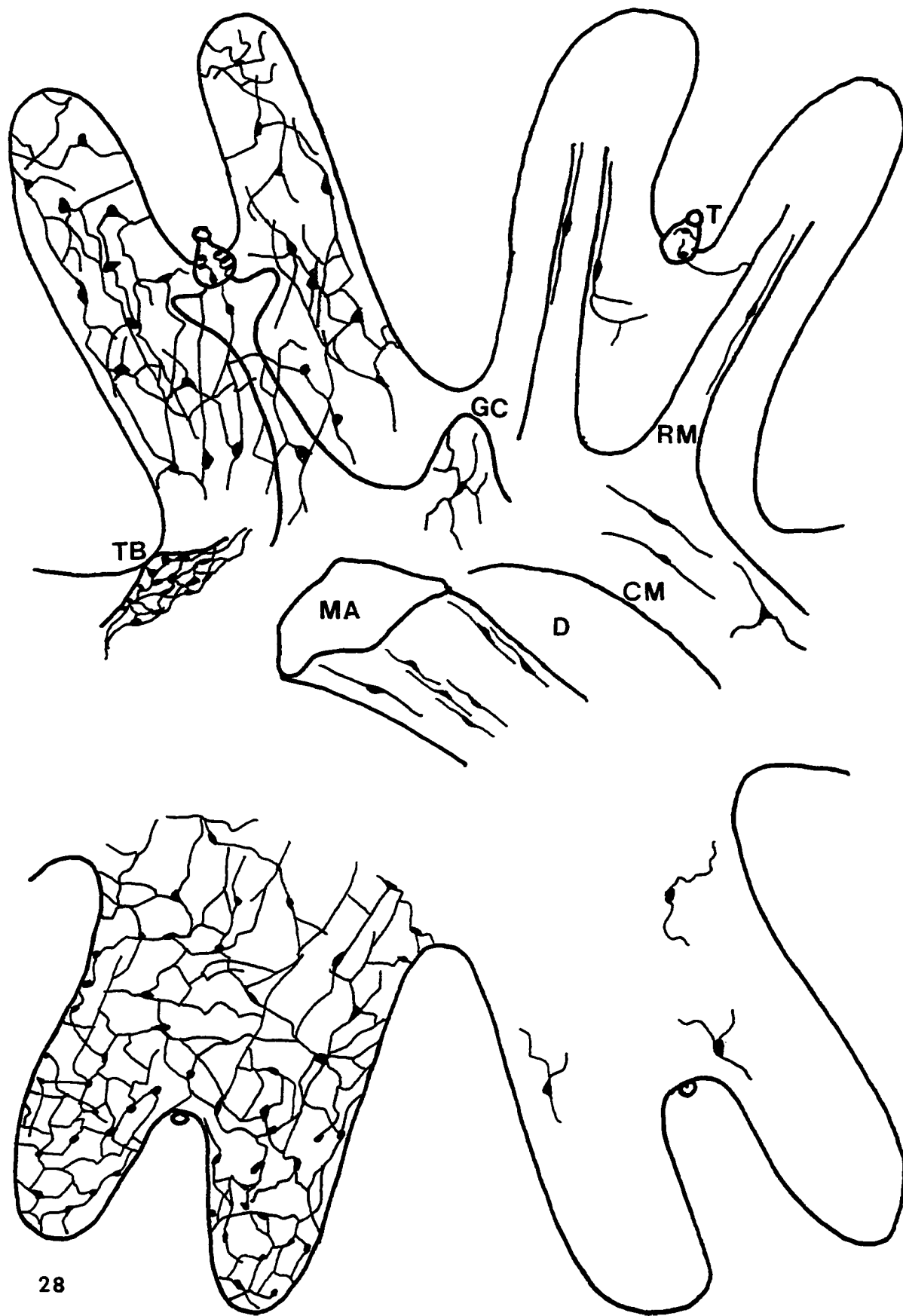


Fig. 29 Electron micrographs of ectodermal cells of the ephyra

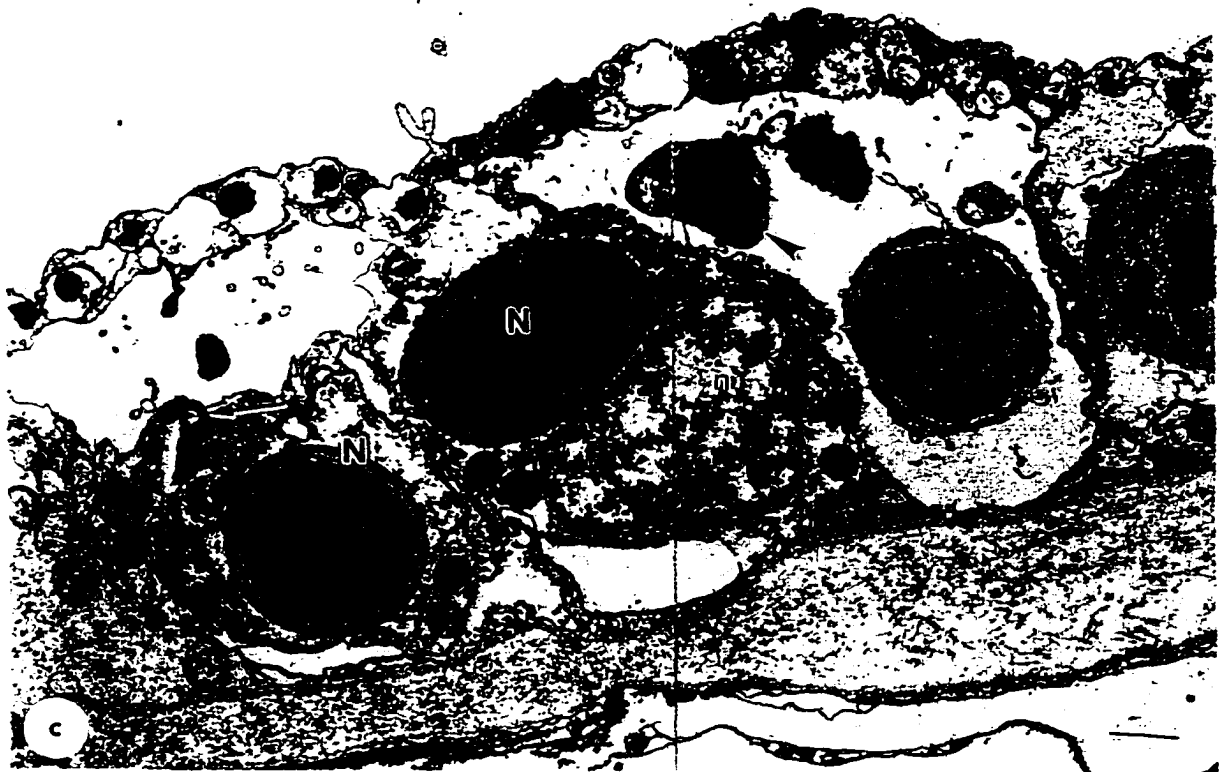
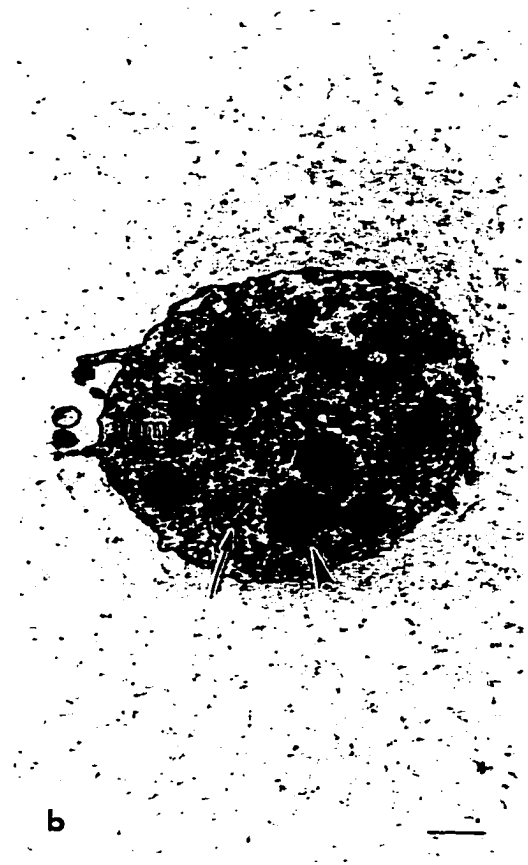
a) Ectodermal cells of the exumbrellar surface frequently have large vacuoles, and may contain inclusions filled with an amorphous substance (arrow).

Nematoblasts (NB) are found at the base of the ectoderm.

b) Amoebocyte in mesogloea contains mitochondria (m), ER (arrow) and densely filled inclusions (arrowhead).

c) Ectoderm of subumbrellar surface contains inclusions filled with an amorphous substance (arrowhead) and others with a more structural content (arrow). Nematocysts (N) are present in varying stages of development.

Scale bar for a)-c) = 1 μ m



ectoderm are the processes of striated muscle cells (Fig. 30a). The latter contain numerous mitochondria next to the muscle. The muscle striations are defined by a 700 nm wide S-band. The A-band is 560 nm wide, and the I- and H-bands 140 nm (Fig. 30b). Sometimes, the processes deeply interdigitate with the underlying mesogloea (Fig. 30c). Processes of smooth muscle cells can also be found and are present in the tips of the lappets, between the radial muscle bands and on the disc and manubrium. Neuronal processes are frequently seen amongst the muscle processes and may contain dense-cored vesicles of approximately 100 nm diameter, with a 40 nm core (Fig. 30c). Sometimes, symmetric synapses are encountered which are characterized by the presence of few, large synaptic vesicles on both sides of the membranes (Fig. 30d). Sensory cells are usually narrower than the surrounding ectodermal cells and are cup-shaped. In the endoderm, the cells can appear in a range of shapes from box-shaped to rectangular, and possess different cell contents (Fig. 31a). They are ciliated cells whose nuclei can be rather large (Fig. 31b). Whereas some of the cells contain a less densely filled cytoplasm, others possess various inclusions which can contain membranous structures or be filled with electron-dense and crystal-like material. Numerous mitochondria and a well developed ER are also frequently encountered. The endoderm of the ephyra also contains neurons and sensory cells. Some fibres contain dense-cored vesicles (Fig. 31c), some of which can be labelled with gold-conjugated anti-FMRamide antibodies (Fig. 31d).

Specificity of Monoclonal Antibody Aa

I harvested 2,192 clones resulting from the fusion and screened them for antibody production. One of the antibodies labelled amoebocytes, two labelled muscle cells, and seven were neuron-specific. All of the antibodies were of the IgM class. The neuron-specific antibodies had similar labelling patterns, and I focused on mAb Aa

Fig. 30 Electron micrographs of muscle and nervous tissue in the ephyra

a) Circular muscle (CM) cells with numerous mitochondria (m); n: nucleus.

Scale bar = 1 μ m

b) Different bands of striation are clearly visible; m: mitochondrion.

Scale bar = 0.5 μ m

c) Circular muscle in cross section. Arrowheads point to interdigitations of muscle process with mesogloea; dcv: dense-cored vesicle in neuronal process.

Scale bar = 0.5 μ m

d) Symmetric neuro-neuronal synapse (arrow); sv: synaptic vesicle.

Scale bar = 0.5 μ m

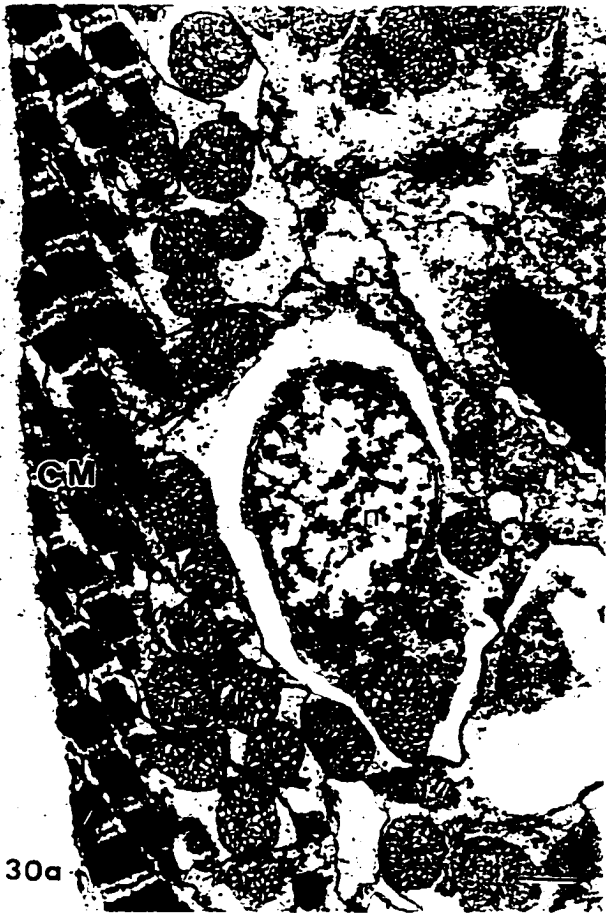


Fig. 31 Electron micrographs of endodermal cells in the ephyra

a) The contents of the endodermal cells can be large and spherical inclusions filled with an amorphous substance (arrow), or they can be more irregularly shaped and contain crystalline and electron-dense material (arrowhead).

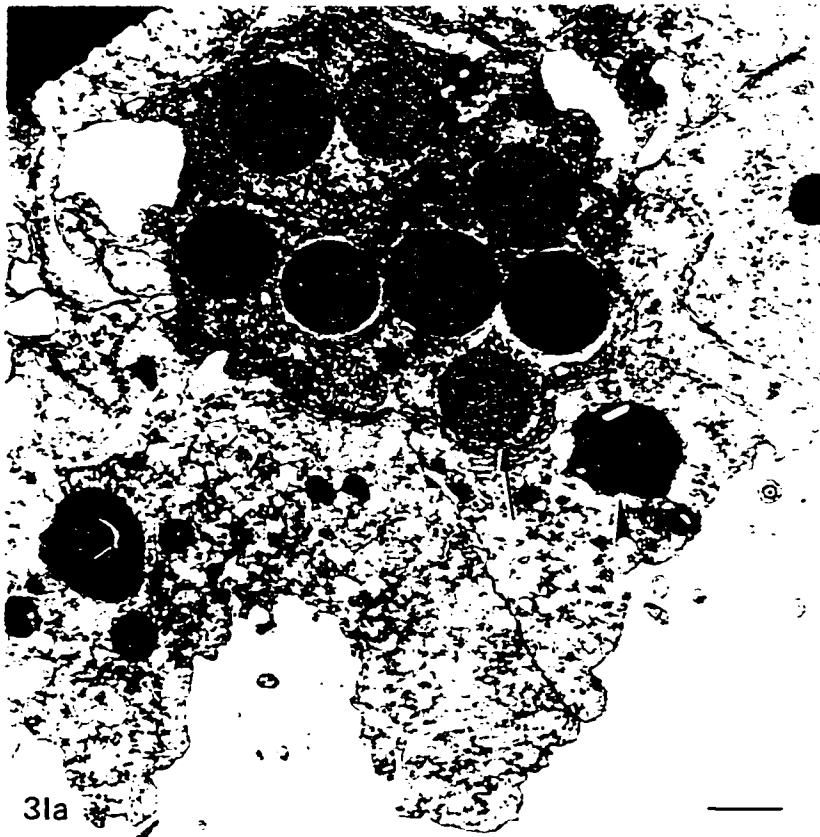
Scale bar = $1\mu\text{m}$

b) The endodermal cells possess prominent nuclei, and can contain large inclusions with membranous contents (arrow). Scale bar = $1\mu\text{m}$

c) Some of the neuronal processes in the endoderm contain dense-cored vesicles (dcv); Scale bar = $0.5\mu\text{m}$

d) Labelling with anti-FMRamide and gold-conjugated secondary antibody shows clusters of gold particles (arrow) on some of the vesicles.

Scale bar = $0.5\mu\text{m}$



because it had a (high) titer of 10^6 when determined by immunofluorescence. MAb Aa labelled neurons in tissue fixed with Zamboni's fixative and paraformaldehyde, but not in glutaraldehyde-fixed tissue. After immunogold-labelling of paraformaldehyde-fixed tissue, a few gold particles were associated with the membrane of a neuronal process (Fig. 32a, b), and clusters of gold particles were found close to the membrane of cell bodies (Fig. 32c, d). A few gold particles were seen in the control, but did not appear to be associated with any structure (Fig. 32e).

MAb Aa labelled neurons in all of the *Aurelia* tested, including those from Japan, the Pacific Northwest, and different locations in California. It is a matter of debate whether the specimens all belonged to the species '*aurita*' (Freya Sommer, personal communication). However, mAb Aa did not label any neurons in any of the other Scyphozoa tested, including *Phacellophora*, which belongs to the same family (the Ulmaridae) as *Aurelia*, as well as *Pelagia*, *Chrysaora*, *Cyanea*, and *Cassiopea*.

Immunoblot analysis showed a single band of approximately 45 kDa when the blot was probed with mAb Aa, but not with a non-relevant antibody (NRA) (Fig. 32f).

Rhodamine B as Indicator of Neuronal Activity

When ephyrae were treated with 0.13M $MgCl_2$ and $10^{-6}M$ FM[D-R]Famide, the number of labelled neurons remained relatively unchanged compared to exposure to seawater alone. 10 animals were used for each treatment. In FSW, 9 animals did not have any stained neurons, and only 1 neuron was stained in the remaining animal. In $MgCl_2$ and FM[D-R]Famide, 8 out of 10 and 5 out of 10 animals, respectively, were unstained, and for both, the average number of stained neurons was 1.1 (Table 6). No neurons were stained when the concentration of $MgCl_2$ was increased to 0.19M.

Fig. 32 Specificity of monoclonal antibody Aa

a) - d) Immunogold-labelling with mAb Aa

a), b) Gold particles (arrow) associated with membrane of neuronal process.

Scale bar in a) = $0.5\mu\text{m}$, in b) = $0.25\mu\text{m}$

c), d) Accumulations of gold particles (arrows) associated with membrane of cell bodies. Scale bar for c) = $0.5\mu\text{m}$, for d) = $0.25\mu\text{m}$

e) Control using NRA; arrow points to gold particles in cytoplasm.

Scale bar = $0.5\mu\text{m}$

f) Immunoblot analysis with mAb Aa shows single lane of approximately 45 kDa (A), but no label with NRA (B). Molecular weight markers are on left for both A and B. Per lane, 15- 20 μl of 0.05-1.3 $\mu\text{g}/\mu\text{l}$ protein were separated.

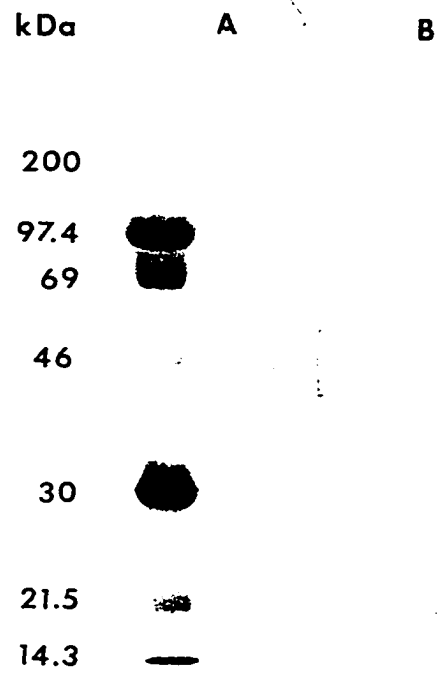


Table 6 Effect of treatment with MgCl_2 and FM[D-R]Famide on number of neurons stained with Rhodamine B

The concentrations were 0.13M MgCl_2 and 10^{-6}M FM[D-R]Famide. Only animals treated with MgCl_2 had neurons stained on the disc, while only animals treated with FM[D-R]Famide had neurons stained in the gastric filaments (GF)

Animal	Total		Arms		Disc	GF
	MgCl_2	FM[D-R]Famide	MgCl_2	FM[D-R]Famide	MgCl_2	FM[D-R]Famide
1	9	2	6	2	3	0
2	2	3	2	3		0
3	0	1	0	1		0
4	0	1	0	1		0
5	0	4	0	0		4
Sum	11	11	8	7	3	4
Average	5.5	2.2	4	1.4	1.5	0.8
Total Average	1.1	1.1	0.8	0.7	0.3	0.4

The number of stained neurons increased 15 times with 10^{-6}M FMRFamide treatment ($P = 0.002$), 38 times when whole *Artemia* ($P = 0.002$), and 11 times when filtered *Artemia* ($P = 0.002$) were added (Table 7, Fig. 33). In all cases, the majority of stained ectodermal neurons was found on the arms (94% for *Artemia*, 87% for FMRFamide treatment). Also, the stained neurons on the arms were mostly bipolar and associated with muscle. Only a few stained neurons were found on the manubrium and the disc. In *Artemia* treated animals, however, 74% of all the stained neurons occurred in the gastric filaments. The ratio of stained neurons from the diffuse nerve net (DNN) versus those of the giant fibre system (GFS) was 1.6:1 for the FMRFamide treatment, but increased to 12.7:1 for the *Artemia* treatment when the neurons of the gastric filaments were counted, and 2.6:1 when they were not included (Table 8, Fig. 34).

Table 7 Effects of treatments on number of neurons stained with Rhodamine B

The treatments consisted of whole and filtered *Artemia*, and FMRFamide ($10^{-6}M$), and were administered to 10 animals each; the data represent the average number of stained neurons per treatment.

CM: circular muscle; Ma: manubrium; GF: gastric filaments.

B: bipolar neurons, except for neurons in the gastric filaments; T: tripolar neurons;

M: neurons stained on striated muscle.

Treatment	Total			Arms			Disc		CM	Ma	GF			
	B	T	M	B	T	M	B	T						
FMRFamide ($10^{-6}M$)	15.2	12	3.3	10.6	13	10	3	10	1	0.7	0.3	0.6	0.5	0.1
<i>Artemia</i> (whole)	38.4	8.9	0.9	7.6	9.4	8.6	0.8	7.5	0.3	0.3	0	0.3	0	28.4
<i>Artemia</i> (filtered)	10.6	6.4	1.4	3.8	6.4	5.4	1	3.8	1.4	1	0.4	0	0	2.8

Fig. 33 Effects of treatments on number of neurons stained with Rhodamine B

The treatments are expressed as a percentage of neurons stained in FSW, and consisted of MgCl_2 (0.13M), FM[D-R]Famide (FMDRFa) and FMRFamide, both at 10^{-6}M , and whole and filtered *Artemia*. For the latter two, two values are given; one where the number of stained neurons of the gastric filaments were included (+GF), and one where they were omitted (-GF).

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

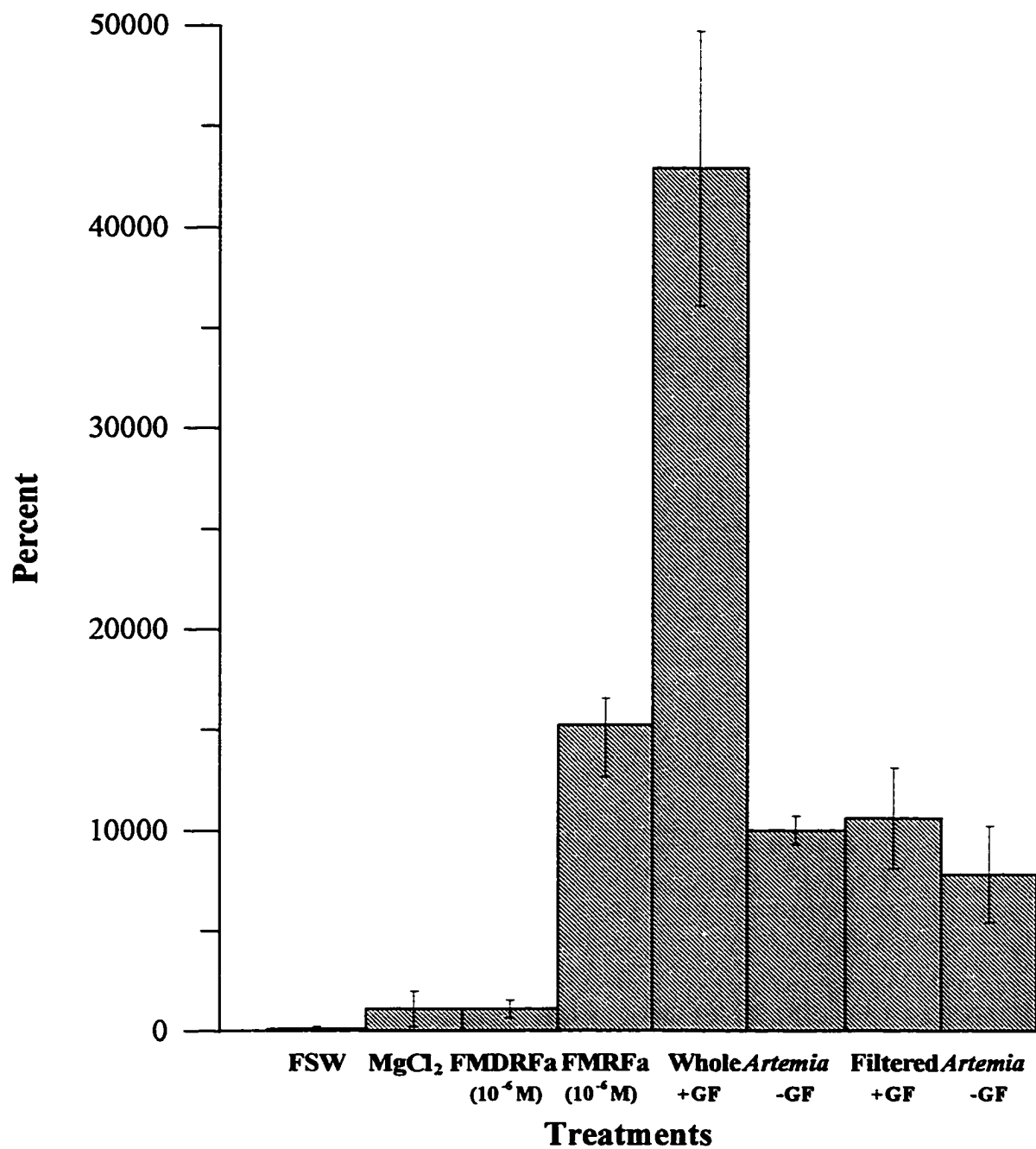


Fig. 33 Neurons stained with Rhodamine B after treatment as a percentage of neurons stained in FSW (FSW=100%)

Table 8 Comparison of neurons stained with Rhodamine B after treatment with whole and filtered *Artemia*, and FMRFamide (10^{-6} M)

For each treatment, the ratio of stained neurons of the diffuse nerve net (DNN) to those of the giant fibre system (GFS) is given.

DNN+GF: neurons of the DNN, including the neurons in the gastric filaments (GF).

DNN-GF: neurons of the DNN, excluding the neurons in the GF.

Treatment		Total	DNN +GF	DNN -GF	GFS	GF
<i>Artemia</i> (whole)	Average	38.4	35.6	7.2	2.8	28.4
	Percent	100	92.71	18.75	7.29	73.95
	Ratio DNN/GFS		12.7	2.6	1	
<i>Artemia</i> (filtered)	Average	10.6	7.8	5	2.8	2.8
	Percent	100	73.58	47.16	26.41	26.41
	Ratio DNN/GFS		2.8	1.8	1	
FMRFamide (10^{-6} M)	Average	15.2	9.3	9.2	5.9	0.1
	Percent	100	61.2	60.5	38.8	0.66
	Ratio DNN/GFS		1.6	1.6	1	

The Swimming Beat

The addition of 10^{-6} and 10^{-7} M FMRFamide caused an increase in the number of swimming beats by about 60%, and the addition of 10^{-6} M glycine by about 35% compared to seawater alone. The number of beats remained relatively unchanged with H_2O and 10^{-7} M FM[D-R]Famide, but decreased by 15% with 10^{-6} M FM[D-R]Famide and by 55% with the addition of *Artemia* (Fig. 35).

Fig. 34 Comparison of neurons stained with Rhodamine B after treatment with whole and filtered *Artemia* and FMRFamide

For each treatment, the total number of stained neurons (=100%) was divided into neurons of the diffuse nerve net (DNN) and neurons of the giant fibre system (GFS).

The treatments consisted of whole and filtered *Artemia*, and FMRFamide (10^{-6} M).

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

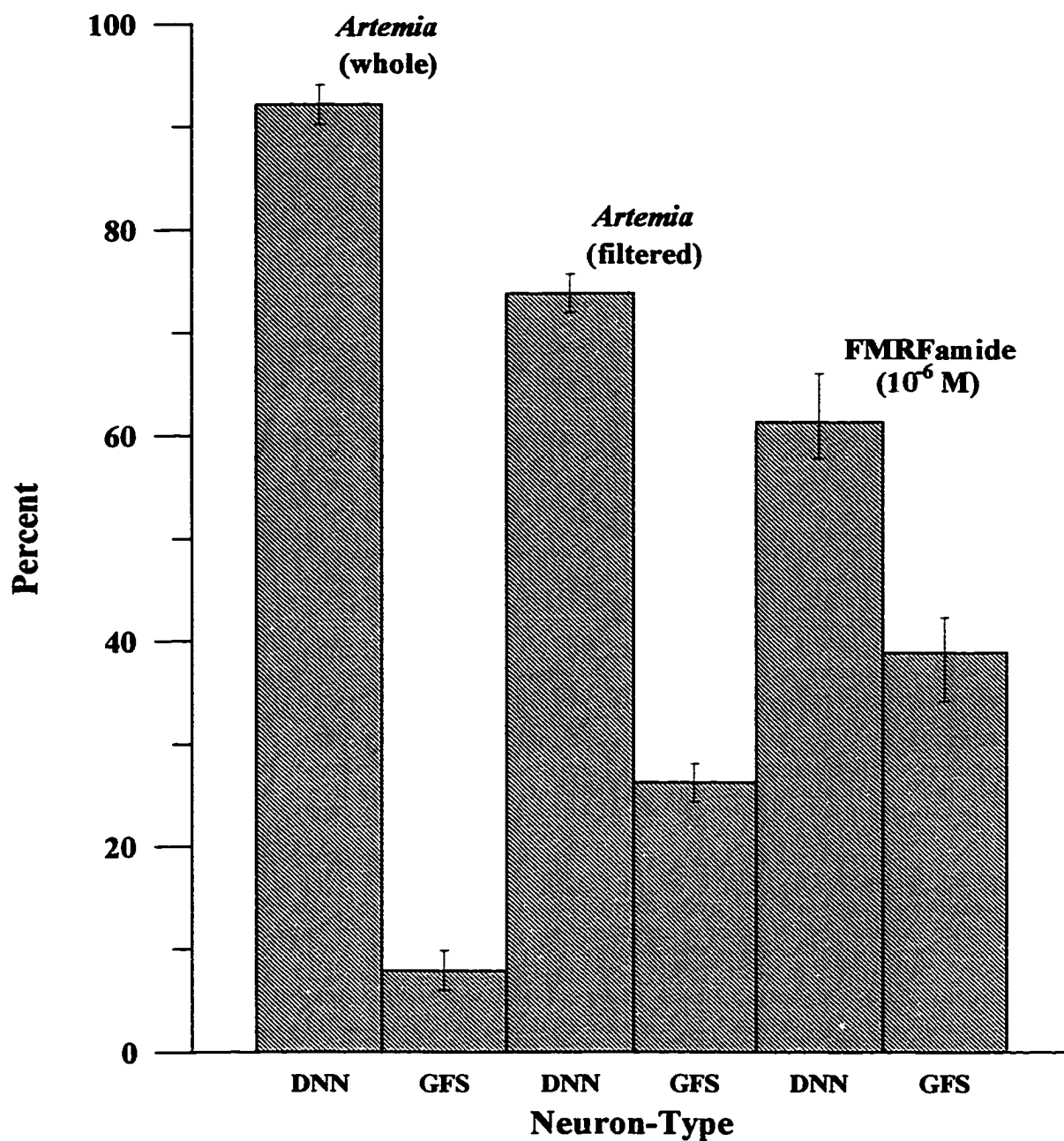


Fig. 34 Comparison of neurons stained with Rhodamine B after treatment with whole and filtered *Artemia* and FMRFamide

DNN: Neurons of the diffuse nerve net

GFS: Neurons of the giant fiber system

Fig. 35 Effects of treatments on swimming beats of the ephyra

5 animals were used for each treatment, and beats were counted for 20 min. each. Bars represent the average number of beats as a percentage of the number of beats in FSW (FSW = 100%)

The standard errors of the mean (vertical bars) were included in order to show the distribution of the means, although nonparametric tests such as the sign test used here do not utilize the estimation of the mean (Zar, 1974).

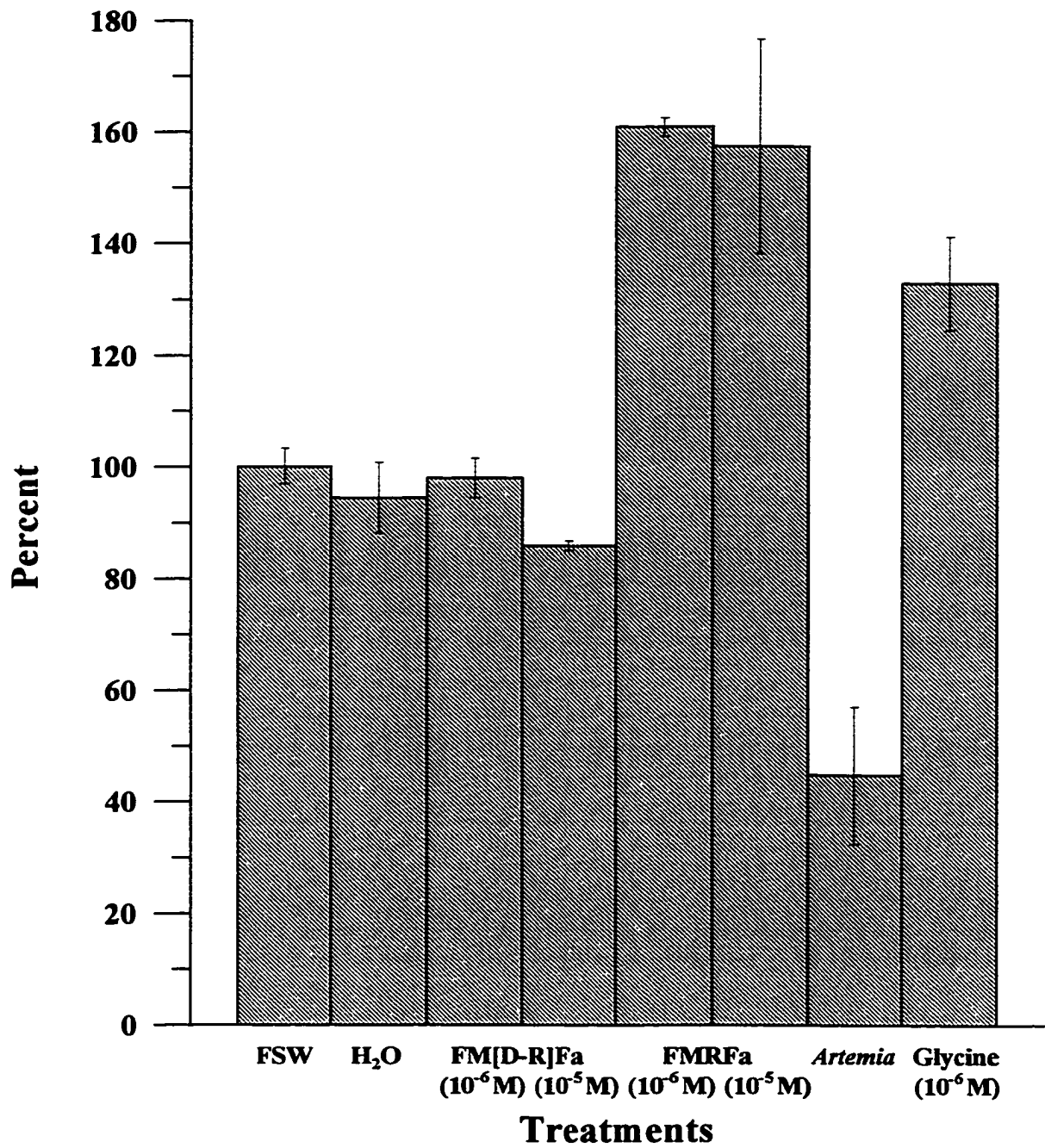


Fig. 35 Effects of treatments on number of swimming beats of the ephyra (Beats/min in % of beats in FSW, FSW=100%)

Discussion

Horridge (1956a), who also worked on the adult jellyfish of *Aurelia* (1954), chose to work on the ephyra in order to investigate the existence of two nerve nets. Already Romanes (1878) had demonstrated in the adult jellyfish that the wave of tentacle contractions traveling around the margin could be distinguished from the muscle contractions of the swimming beat, and that the tentacle wave could initiate a contraction wave when it reached a tentaculocyst. The giant fibre system described by Schäfer (1928) had been recognized as propagating the contraction wave, but there was insufficient anatomical evidence for a second net propagating the tentacle wave. The ephyra, which demonstrates two distinct behaviours (swimming and feeding response), presented an ideal system in which to study both physiologically and histologically the existence of two overlying nerve nets. Indeed, Horridge's (1956a) methylene blue preparations revealed the giant fibre system and the diffuse nerve net. Given the dimensions of the neurons involved, the term 'giant' is based on functional rather than anatomical bases, since the diameter of the fibres (less than $1\mu\text{m}$) does not compare to giant fibres in other phyla (in the earthworm, for example, the dorsal lateral and median giant fibres reach diameters of up to $70\mu\text{m}$ and $100\mu\text{m}$, respectively).

In this study, staining with methylene blue confirmed Horridge's (1956a) results, which could be expanded with results from antibody-labelling. Methylene blue visualized members of the GFS, DNN, and neurons in the tentaculocyst. Labelling with anti-FMRamide revealed an extensive nerve net consisting of both sensory and nerve cells covering all surfaces. The appearance of the nerve net is very similar compared to that of other Scyphozoa (Anderson, 1992). MAb Aa labelled isolated nerve cells which resembled bipolar giant fibres when associated with striated muscle of either the radial or circular muscle bands. In addition, mAb Aa labelled multipolar cells on the manubrium, exumbrella, and in the endoderm. In contrast, endodermal

cells in the adults of *Phacellophora* and *Cyanea* (Passano and Passano, 1971) are mostly bipolar, with most fibres ending on other neurons and only few free nerve endings. In *Aurelia*, it appears as if neuro-neuronal contact of mAb Aa-positive neurons is mostly established during the development from the ephyra into the juvenile and adult, when the number of neurons as well as neurite length increase.

Because integration of nervous information occurs in the tentaculocysts, and because Horridge's (1956a) scheme provides a plausible explanation for how this could be accomplished, I have attempted to interpret the identification of labelled neurons according to Horridge. In his scheme, input to the tentaculocyst from the diffuse nerve net is received by the "d" cells, and output from the tentaculocyst to the giant fibres is provided by the "a" cells. Both types of cells are synaptically connected to the "b" cells, which also synapse on each other, and "c" cells synapse on and synchronize the "a" cells on either side of the tentaculocyst. Both anti-FMRFamide and Aa antibodies labelled cells in the tentaculocyst corresponding to the cell types identified by Horridge, although mAb Aa labelled one type of neuron which does not easily fit into Horridge's scheme of "a" to "d" type neurons. The location of this neuron at the base of the tentaculocyst corresponds to that of the type "a" cell, but the fibres of the latter either extend to the radial muscles or ascend into the tentaculocyst. According to the diagrams provided by Horridge (1956a), only fibres of type "c" neurons extend contralaterally as far as the mAb Aa-positive neuron does, although they occur near, but never directly at the base of the tentaculocyst. Of the two cell types, "a" and "c", the mAb Aa-positive neuron best fits the description of the type "c" cells, given that Horridge observed variations in the location, as well as in the shape of the cell bodies and the extent of the branching patterns of the fibres. On the other hand, Horridge cautioned that he may not have observed all cell types; perhaps the mAb Aa-positive neuron is one of the ones he missed. In addition to the exact number of neuronal cell types present in the tentaculocyst, another question that remains largely unanswered by

Horridge's scheme concerns the direction of synapses in the tentaculocyst, i.e. which cells are presynaptic, and which cells are postsynaptic? There is physiological evidence of DNN action on the GFS (Horridge, 1959; Passano, 1965) and accordingly, the "d" cells must be presynaptic and the "a" cells postsynaptic. On the other hand, Horridge's (1956a) scheme allows for any of the cell types to be both presynaptic and postsynaptic, and ultrastructural evidence indicates that most of the synapses in the tentaculocyst are symmetrical, *i.e.* bidirectional (Horridge and Mackay, 1962). As yet, there is however no physiological evidence supporting GFS action on the DNN.

Horridge (1956a) also mentioned the symmetry of the tentaculocysts which, as well as the arms, show a marked bilateral symmetry in an otherwise radially symmetrical animal. As far as elements of the nervous system are concerned, however, this symmetry is functional rather than structural, *i.e.* the halves of either the tentaculocyst or the arms are not mirror images of one another as evidenced by differences in the distribution, shape, branching patterns and, in some cases, the number of cells. The mAb Aa label reflects this pattern, which can also be observed when comparing octants to one another, *e.g.* the mAb Aa label is never identical in any given pair of arms.

The function of the mAb Aa-positive neurons which are associated with muscle cells is most likely innervation of the latter, but given that those neurons more closely resemble neurons of the GFS in that they are stouter and the fibres are straighter compared to DNN neurons, the appearance of mAb Aa-positive neurons on the exumbrellar surface and in the endoderm in this context is puzzling, as is the fact that neurons which seem to be part of the giant fibre system are associated with smooth muscle. Either I am wrong in assuming that some of the mAb Aa-positive neurons belong to the GFS, or the DNN and GFS need to be subdivided according to functional, morphological, and immunocytochemical criteria. Clearly all of the mAb Aa-positive neurons, regardless of function, share a common epitope. While the

orientation of the neurons along the muscles could indicate that the epitope is involved in neuronal guidance, the fact that the number of neurons increases as the animals develop and achieve their highest concentration in the adult suggests that the epitope is not present only temporarily. The orientation of the cells, then, could be unrelated to the expression of the epitope, and could be due to muscle-neuron interactions, with the muscles providing the guidance for the developing fibres. Carlberg *et al.* (1995) reported taurine-like immuno-reactivity in ecto- and endodermal neurons of *Cyanea*. However, these neurons were also labelled with anti-FMRFamide, so it is unlikely that he described the same type of neurons that are labelled with mAb Aa.

It is interesting that the ephyrae develop from disc-shaped structures and develop into disc-shaped adults, but possess eight arms in the interim. Consequently, a portion of the force enabling swimming results from the beating of the arms, whose contribution towards swimming in older animals is negligible. Rather, in the adult, swimming beats are a result of the contractions of the circular muscle band. In the ephyra, however, the radial muscle bands play a much more important role during swimming, and the input received from the tentaculocysts by the giant fibres innervating the radial muscle is directly translated into swimming action. The fact that the arms can move independently contributes to the rate of food-capture and its transport to the mouth. I have only used and observed *Artemia* as the food of choice. *Artemia* has proven to be an adequate food source and is being used successfully for all stages of the life cycle of *Aurelia* at the Monterey Bay Aquarium. However, animals at the latter facility tend to be consistently larger and healthier than animals maintained in the seawater system of the University of Victoria, even when provided with the same quality and quantity of food. The smaller size of the ephyrae could explain their limited success in capturing *Artemia* at a higher rate.

Fluorescent dyes have been used successfully to stain living cells. Dyes such as rhodamine 123 stain mitochondria (Johnson *et al.*, 1980), and have been employed to

detect presynaptic nerve terminals (Yoshikami and Okun, 1984), where mitochondria are abundant. Of the rhodamine compounds tested it has become apparent that only those that are positively charged at physiological pH, namely rhodamine 123, 6G and 3B, are able to stain mitochondria specifically whereas uncharged rhodamines such as rhodamine B do not. The results suggest that the cationic rhodamine molecules are selectively accumulated by the mitochondria of living cells, and that the mitochondria-specific interaction of such molecules is dependent on the high trans-membrane (inside negative) potential maintained by functional mitochondria, since dissipation of the potential eliminates the selective mitochondrial association of these compounds. Rhodamine B, on the other hand, probably enters cells via synaptic vesicle recycling, which has been demonstrated at the frog neuromuscular junction (Betz and Bewick, 1992). In *Aurelia* that are immersed in Rhodamine B, the latter possibly arrives at the cells of the ectoderm via the gastric cavity and the mesogloea, as Lane *et al.* (1965) showed that the mesogloea of *Aurelia* is loosely organized and permeated by extracellular fluid.

Rhodamine B appears to be a reliable indicator for neuronal activity in the ephyra, given that comparable results could be obtained in the replicates, and few cells were stained in animals treated with $MgCl_2$, which reduces or inhibits transmitter release. Rhodamine B also showed that the sensory cells in the gastric filaments occur at a much higher number than was indicated with any of the other labels or stains. The cells could be involved with the regulation of nematocyst discharge and/or the release of digestive enzymes. While the role of the nervous system in nematocyst discharge is under debate, Westfall (1988) proposed that mechanical and chemical stimuli trigger nematocyst discharge either directly or via sensory and ganglion cells. The percentage of stained sensory cells in the gastric filaments was highest in animals treated with whole rather than filtered *Artemia* (74% compared to 26%), suggesting that both mechano- and chemosensory cells were stimulated in the former, while the latter

mainly responded to chemical stimuli. The fact that less than one percent of cells were stained in the gastric filaments of animals treated with either FM[D-R]Famide or FMRFamide, which increases the number of swimming beats, indicates that neither acts as a food substance. When the gastric filaments are not taken into consideration, the majority of stained cells in both *Artemia*- and FMRFamide-treated animals were bipolar neurons on the radial muscles. The fact that the DNN is involved in the feeding response and the GFS in the swimming movements is reflected in the ratio of stained DNN to GFS neurons. In animals treated with whole and filtered *Artemia*, the ratio is 12.7 : 1 and 2.8 : 1, respectively, while it is 1.6 : 1 in FMRFamide-treated animals. The latter is partly due to an increased number of stained GFS neurons, but also reflects the reduced number of stained DNN cells in the gastric filaments. The fact that only few cells were stained in animals in FSW was somewhat surprising, as I would have expected the number to be higher (at least when compared to MgCl₂-treated animals), due to spontaneous activity of the animals. Perhaps the results are due to the fact that the animals were kept in the dark for part of the treatment, because light affects pulsation frequency and perhaps other swimming parameters (Mackie *et al.*, 1981), and other external stimuli were absent.

As with the mAb Aa-labelling, Rhodamine B stained cells were not distributed in a symmetrical fashion. Given that, during feeding, different arms are active at a different rate, depending on their encounter with food particles, this seems a reasonable result. However, during swimming, I would have expected the staining to be more evenly distributed. One has to keep in mind, however, that Rhodamine B tends to fade fast, and I could have overlooked a number of previously stained cells.

As far as the effects of treatment on the swimming beat are concerned, the latter was increased by FMRFamide and glycine, but decreased by *Artemia* filtrate. The response to FMRFamide is in accordance to its action in other invertebrates (McFarlane *et al.*, 1989). In sea anemones, FMRFamide increases the frequency of contractions,

and in *Helix aspersa*, it causes rhythmicity in the pharyngeal retractor muscles (Lehmann and Greenberg, 1987), and there is evidence that it acts directly on the muscles. The effect of the *Artemia* filtrate is in agreement with the fact that feeding *Aurelia* either stop or decrease their swimming activity. The fact that glycine increased rather than decreased swimming activity seems to indicate that it does not act as a food substance in this case. Perhaps glycine has a neuromodulator/transmitter function in *Aurelia*. However, glycine is generally the most concentrated amino acid dissolved in sea water, and Schick (1975) showed that the effect of starvation, which resulted in a reduced number of strobilating *Aurelia*, could be overcome by exposing the animals to 10^{-6} M glycine, suggesting that it does represent a supplemental energy source.

Although I attempted to select animals that were of the same developmental stage, there may have been differences between different set-ups since the experiments had to be carried out on different days, depending on when animals were available in sufficient numbers. In addition, swimming activity varies between animals, and activity levels may also have been influenced by whether or not the animals had been feeding prior to the exposure to the treatments.

4. GENERAL DISCUSSION

In the planula of *Aurelia*, only few neurons and sensory cells are labelled with anti-FMRamide, and the cells and their fibres form a nerve net just prior to the onset of metamorphosis. The level of complexity of the nervous system, as well as the overall complexity of this stage, is much reduced compared to other Cnidaria, in that several different cell types have been reported for both the Hydrozoa (Martin *et al.*, 1983) as well as the Anthozoa planula (Chia and Koss, 1979). Metamorphosis in *Aurelia* planulae can be induced by iodine, thyroxine, and retinoic acid. Although the

effectiveness of the former two compounds, which also induce strobilation (Spangenberg, 1967), has been known, the effect of retinoic acid had previously not been demonstrated. FMRFamide, which, as suggested by Brumwell and Martin (1996), is necessary for metamorphosis to occur in hydrozoan planulae, reduces the rate of metamorphosis in *Aurelia*.

In the scyphistoma, anti-FMRFamide labels a net of neurons and sensory cells on the oral disc and the tentacles, fibres associated with the muscle bands, and single or pairs of fibres traversing the length of the column. Only one conducting system appears to be present (Chia *et al.*, 1984), of which the FMRFamide-positive neurons form a subset. The level of complexity of the nervous system is reduced compared to both Anthozoa and Hydrozoa. In the Anthozoa, several members of the RFamide family have been identified (Grimmelikhuijzen *et al.*, 1989), some of which appear to label different neuronal populations. In addition, the presence of dopamine, noradrenaline, adrenalin, and serotonin has been demonstrated (Anctil, 1989), and three separate conducting systems have been reported (McFarlane *et al.*, 1989). In the Hydrozoa, the anti-FMRFamide label extends to the body column, where a plexus of fibres is labelled, and in some cases reveals a nerve ring on the oral disc (Grimmelikhuijzen *et al.*, 1989). In addition to the presence of catecholamines and serotonin, cholinergic sensitivity has been suggested (Scemes, 1989), and vasopressin-like immunoreactivity has been reported (Koizumi and Bode, 1991). According to McFarlane *et al.* (1989), neural organization of the Anthozoa more closely resembles that of the Scyphozoa than the Hydrozoa, but they compare it to the nervous system of the jellyfish and not the scyphistoma. As far as overall complexity of the scyphistoma is concerned, it takes a position between the Anthozoa and Hydrozoa, *e.g.* as supported by the compartmentalization of the gastric cavity.

In the jellyfish, two different nets have been identified, *i.e.* the giant fibre system and the diffuse nerve net. Integration of information of the two systems takes

place in the tentaculocysts, where input from the diffuse nerve net is received (by the "d" cells) and output to the giant fibres is provided (by the "a" cells). As yet, there is no evidence of GFS action on the DNN. Anti-FMRamide labels a subpopulation of neurons of the DNN which form a nerve net covering the subumbrellar and exumbrellar surfaces. MAb Aa labels a subpopulation of neurons which appears to include cells of both the giant fibre system as well as the diffuse nerve net. Only few and scattered neurons are labelled in the ephyra, but the labelled neurons increase in number during development and form an interconnected population of cells in the adult jellyfish. Compared to the nervous system of hydrozoan medusae, the jellyfish are less complex in that they lack concentrations of neurons as they are found in the inner and outer nerve rings. In addition, several different subsets (at least six) of neurons have been reported (Mackie, 1989), two in the outer nerve ring alone (Spencer and Arkett, 1984), as well as different types of giant axons (Mills *et al.*, 1985), some of which are able to conduct two different sorts of impulses (Mackie and Meech, 1985). Also, gap junctions are present in the Hydrozoa, but have not been observed in the Scyphozoa (Mackie *et al.*, 1984). In the Anthozoa, Germain and Anctil (1996) presented evidence for the existence of intercellular coupling and a connexin-like protein in the endoderm of *Renilla*, although they did not observe any conventional gap junction plaques.

As far as overall complexity is concerned, one of the major differences is the absence of a velum in the Scyphozoa; the developing hydrozoan medusae, however, already resemble the adult and do not undergo a transformation from an ephyra-like stage.

While the Scyphozoa, on one hand, are the least complex compared to other coelenterates, the structure of the nervous system, at least, proved to be more complex than anticipated. It appears as if throughout the development of the nervous system, new neurons are added, while neuronal cell death does not appear to play a role. (Partial) atrophy of some cells does occur, as seen in the muscle cords of the strobila,

but there seems to be no loss of neuronal cells. The fact that subsets of neurons can be identified provides support to the suggestion by Passano (1982) that more than just the two nerve nets (DNN and GFS) are present. The need for the latter might be indicated on the subumbrellar surface which carries the muscles that are involved in different behaviours, but on the other hand, the existence of two nerve nets on the exumbrellar surface is most puzzling. Do they both provide innervation for the nematocysts and/or transmit information from the sensory cells?

MAB Aa does not label neurons in the planula or the scyphistoma, indicating that either the neurons themselves did not exist in those stages, or that they did not possess the specific antigenic site for labelling to occur. Here, then, along with the first appearance of striated muscle cells coinciding with the free-swimming life style, is the 'birth' of a new type of neuron, which was previously undiscovered. At the ephyra-stage, the mAb Aa-positive neurons are not distributed evenly and symmetrically, but appear scattered over the surfaces, and more and more neurons are labelled as the animals develop. Nevertheless, because of the simultaneous appearance of the striated muscles and the mAb Aa-positive neurons, it is tempting to try and establish the identification of the mAb Aa-positive neurons through a link between the two, either functional, *e.g.* that the mAb Aa-positive neurons innervate the muscle, or developmental, *e.g.* that they are guided by information provided by the muscle. However, the existence of mAb Aa-positive neurons on the exumbrella and in the endoderm do not advocate a link to muscle cells for those neurons. MAB Aa not only specifically labels neurons in the jellyfish stages alone, it also (regrettably) does not label neurons in any other Scyphozoa. Herein lie both the advantage and disadvantage of monoclonal antibodies. In order to obtain antibodies specific for neurons, the production of monoclonal antibodies was indicated since it proved impossible to obtain a pure enough preparation of neuronal tissue, which would have been necessary for the

production of antisera. The latter, however, might have increased the probability of cross-reactivity with neurons of other species.

The reduced complexity of the nervous system of the Scyphozoa compared to other Cnidaria is in line with their overall reduced complexity, and it has been proposed (Werner, 1975) that they resemble most closely the ancestral coelenterate. Haeckel (1874, 1884) and Lankester (1877) agreed that the original diploblastic metazoan ancestor was derived from colonial choanoflagellates, even though they disagreed on the manner of endoderm formation and consequently on the matter of a hollow versus a solid organism. They agreed also that the most likely extant candidates to approximate the ancestral condition are the coelenterates. Werner (1975) argued that the ancestors of the coelenterates were solitary, tetramerous and sessile organisms resembling the extinct Conulata, whose living relatives could be coronate scyphozoans. The Anthozoa were an early offshoot from this stem form, from which the Scyphozoa and then the Hydrozoa were derived. Werner reversed the evolutionary sequence Hydrozoa-Scyphozoa-Anthozoa proposed by Hyman (1940), who envisioned medusoid ancestors for the coelenterates. Assuming that organizational simplification represents the ancestral and not a derived condition, there are a number of arguments supporting Werner's view. Compared to both Hydrozoa and Anthozoa, Scyphozoa in general have fewer nematocyst types, and none of the latter are exclusive to the Scyphozoa. About 14 different major classes of nematocysts have been described so far. Most of them are divided into different types, accounting for approximately 30 different types total. The Scyphozoa only possess stomocnidae, *i.e.* nematocysts whose thread has a terminal opening, and only two classes (isorhizae and microbasic euryteles) and four different types are represented. In the Hydrozoa and Anthozoa, 11 classes and 23 types, and 5 classes and 6 types are present, respectively. Of the latter, 17 types are exclusive to the Hydrozoa, and four types are exclusive to the Anthozoa. Furthermore, two of the anthozoan classes are markedly different from all the other nematocysts: in

the capsule structure and electron density of capsule contents (spirocysts) and the manner in which the thread is stored in the capsule (accordion-like rather than coiled in ptychocysts). Thus, the distribution of nematocysts could be accounted for by a scyphozoan-like coelenterate ancestor and an early anthozoan offshoot from the stem form.

In the Anthozoa and Scyphozoa, the gonadal tissues are of endodermal origin, while they are ectodermal in the Hydrozoa, arguing for a closer relation between the former and a later phylogenetic origin of the latter. This point is also supported by the fact that structures such as conventional gap junctions are only found in the Hydrozoa, but have not been observed in either Anthozoa or Scyphozoa. The evidence for intercellular coupling and a connexin-like protein in *Renilla* (Germain and Anctil, 1996) could indicate that they are also present in the Scyphozoa and have simply not been found yet; however, it is also possible that the Scyphozoa have remained closer to the (gap junction-less) ancestral condition.

Evidence that the Anthozoa occupy a basal position compared to the other classes was provided by Bridge *et al*, (1992), who showed that the Scyphozoa and Hydrozoa possess linear mitochondrial DNA, while it is circular in the Anthozoa. Given the uniformity of this character throughout the metazoa, the data would support the view that the coelenterate ancestor was sessile. Whether this ancestor more closely resembled the extant Anthozoa or Scyphozoa remains to be seen, however, the present study has shown that, at least as far as the nervous system is concerned, the Scyphozoa have the least complex organization of the Cnidaria. Taken together with the reduced complexity of the Scyphozoa with respect to the organization of the life cycle stages, they make a good candidate to study the ancestral condition. They also provide a number of advantages as far as experimental set-ups are concerned. The scyphistoma can be maintained in much higher numbers than, *e.g.* a sea anemone, and given that

strobilation can be induced, can provide a virtually endless number of jellyfish (unless, of course, one is plagued with an uncooperative sea water system).

As far as the objectives are concerned that I set out to fulfill at the beginning of this study, I have documented the organization of the nervous system in each stage of development and have been able to distinguish different subsets of neurons. I have also investigated the involvement of the nervous system in developmental processes, and compared *Aurelia* to other Scyphozoa. However, although I have examined each stage of the life cycle, a closer examination of more transitional stages, *e.g.* from the planula to the scyphistoma, would undoubtedly provide more insights. For example, what is the fate of the FMRFamide-positive neurons in the anterior region of the planula? Do they become part of the system of fibres covering the muscle bands, or are they represented in the single fibres traversing the column? Where do new neurons come from during development, *e.g.* do amoebocytes differentiate into neurons? The effects of thyroxine and retinoic acid on metamorphosis have been demonstrated, and it would be interesting to identify and compare their receptors to the ones found in vertebrates, where both compounds play biologically significant roles. The fact that Rhodamine B consistently stains active neurons proved to be very informative, even though stained neurons are bleached easily and one runs the risk of missing stained cells because of this. However, the method was unsuccessful in the planula as well as the scyphistoma, and although I have tried modifications of the procedure, perhaps further modifications are necessary. Also, while the Aa monoclonal antibody provides a powerful tool in the identification of neuronal subsets, the characteristics of the molecule and individual epitope that mAb Aa recognizes are still elusive. Attempts at sequencing have failed, probably because of a N-terminal block, and further efforts are needed to identify the molecule. Also, I used unfixed ephyral tissue as the immunogen, which was probably, at least partly, the reason that the immunogold procedures were unsuccessful on glutaraldehyde-fixed tissue, which would have provided a better preservation of

ultrastructural details. At the moment, knowledge of the apparent molecular weight alone and the potential association with membrane make it difficult to even speculate on the nature of the antigen. It would also be interesting to attempt to generate antibodies using tissue from the scyphistoma (or the planula) in order to examine the existence of different subsets of neurons.

Aurelia proved to be a very interesting study object, much more complex than I had anticipated, and not only provided interesting, but also esthetically pleasing results (thanks, at least in part, to mAb Aa, the first, and so far only, neuron-specific monoclonal antibody for Scyphozoa).

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