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Light and electron microscopical studies on the development of the neural complex of *Ascidia mentula* (müller, 1776) (urochordata – ascidiacea)

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Abstract

Specimens of *Ascidia mentula* were collected from the sub-littoral region of the Mediterranean Sea during October 2008, then artificial heterologous insemination was carried out. The nervous system appeared as a neural plate (neuroectoderm) in the late gastrula stage. Invagination of this neural plate proceeds. Closure of the neuropore takes place by fusion of the medullary folds. The curvature of the neural plate is initiated by the outgrowth of the tail in the tail-bud stage. The neural tube is slightly dilated anteriorly to form the cerebral vesicle. Pigmented granules are deposited in the interior of certain cells of the dorsal wall of the cerebral vesicle. At this early stage, the otolith (the ocellus) is represented by scattered rounded pigment granules lying in several rows inside the cells of the dorsal wall of the cerebral vesicle. Another aggregation of pigment granules forms the statolith. As the otolith granules increase in number, they become very much smaller, and then lie entirely at the inner free extremities of the cells. The statolith granules do not tend to increase in number, but retain their original size until they fuse together. The nervous system of the newly hatched larval stage consists of a perfectly closed tube with a central cavity. The cerebral vesicle contains an ocellus and a statolith. These pigmented granules disintegrate gradually as metamorphosis proceeds. As the tail phagocytosed completely, the neural complex appeared macroscopically as a nervous mass having two anterior, two posterior and one median nerve trunks. The differentiation of the neural complex into a dorsal nerve ganglion, a ventral ovoid neural gland and a hypophysial duct occurs after metamorphosis of the swimming tadpole. The nerve cell bodies inside the ganglion are relatively small and could be classified ultrastructurally according to their cytoplasmic contents; into three types which are cells having abundant mitochondria and no granular components, cells having numerous electron-dense granules and cells having electron-translucent granules. Many glial cells, neurosecretory granules, Golgi complex, rough endoplasmic reticulum, mitochondria and secondary lysosomes are demonstrated inside the nerve cells of the ganglion. The nerve trunks consist of mylinated and non-mylinated nerve fibres. An epithelial phase having mononucleated and binucleated cells and a vacuolated lacunated phase with degenerated cells and secretions which can be observed inside the neural gland. The neural gland epithelium appeared as a simple squamous, cubical or pseudostratified layer.

Keywords: Neural plate - Otolith - Statolith - Nerve ganglion - Neural gland - Glial cells - Bodies of neurons- Gland cells - Mitochondria - Secondary lysosomes - Golgi complex - Rough endoplasmic reticulum

1. Introduction

The Mediterranean Sea constitutes a hotspot of marine diversity, with species widespread across a large number of communities (Bianchi and Morri, 2000). Sea squirts, or ascidians, have a metamorphic development, and the adult stage arises from a transient free-swimming tadpole larva (Saad, 2002; Hofmann, *et al.*, 2008 ; Saad and Hamed, 2009). Ascidian larvae have been promoted by enthusiasts as a virtual chordate. In the larvae of the sea squirt *Ciona intestinalis* and *Halocynthia roretzi*, the embryonic cells or blastomeres are about one third of the somatic cells in the nematode *C. elegans* (White *et al.,* 1999). Like the latter too, the cells of the ascidian larva have a pattern of cell lineage that is claimed to be invariant (Satoh, 1994; Gilbert and Raunio, 1997). These features are especially well established in the common sea squirt *C. intestinalis*. There is, in fact, already sufficient interest in the ascidian tadpole larvae and its tiny central nervous system (CNS), especially in *Ciona*. The larva in *Ciona* is endowed with what has been characterized rather loosely as a chordate brain in miniature (Meinertzhagen and Okamura, 2001; Meinertzhagen *et al.,* 2004; Dolcemascolo *et al.,* 2005). This feature alone does not distinguish the genus *Ciona* from the larvae of other ascidians. Analysis of the CNS in *Ciona* is facilitated by two additional features. First, the draft sequence of the *Ciona* genome has been

released (Dehal *et al.,* 2002). Second, *Ciona* embryos DNA can be analysed by means of electrophorases (Corbo *et al.,* 1997), providing a way to analyze gene action in the developing nervous system.

Despite the existing knowledge about the larval CNS (Katz, 1983 ; Nicol, and Meinertzhagen, 1991 ; Meinertzhagen *et al.,* 2004), many questions remain concerning the number and lineage of its cells. Incomplete cell constancy is seen in many animal species in different groups (Van Cleave, 1932). Neuron numbers in ascidian brains are closely determined than in much larger vertebrate brains. Moreover, the number of their types or classes which requires developmental and genetic specification was studied. Among various examples considered by Williams and Herrup (1988), variation in total cell number is greatest among populations of neurones with parallel arrays, whereas there are numerous cases of identified neurones, mostly in invertebrates, in which there is no variation.

The dorsal tubular nervous system is derived in the ascidian embryo from the rolling of a flat neural plate into a hollow neural tube (Katz, 1983 ; Nicol and Meinertzhagen, 1988; Nishida, 1986 ; Bertrand *et al*., 2004 ; Brown *et al.*, 2004). It occurs in a manner resembling that seen in amphibian embryos and thus closely conserved during vertebrate neurogenesis. In the ascidian *Ciona intestinalis* the central nervous system is composed of fewer than 400 cells, and is formed without extensive cell migration or cell death (Nishida, 1986; Gilbert and Raunio, 1997; Meinertzhagen *et al*., 2004). With their chordate affinities, ascidian larvae have been widely called prototypes of vertebrate neurogenesis (Dolcemascolo *et al.,* 2005). The propitious features of the ascidian larval nervous system are summarized by some authors (Meinertzhagen and Okamura, 2001 and Meinertuhagen *et al.,* 2004; Dolcemascolo *et al.,* 2005). With the release of its draft genome (Dehal *et al.,* 2002), *C. intestinalis* is rapidly securing a position as a model genetic organism (Satoh *et al.,* 2003) and numerous genes specific to the nervous system have already been characterized (Satou *et al.,* 2002). The central nervous system of the ascidian larva displays overall homology to the vertebrate nervous system, but is remarkably simple and consists of around 100 neurons and 250 glial cells. These few neurons however, constitute a sufficiently complex CNS to support swimming and enable the ascidian tadpole to sense and respond to light, hydrostatic pressure, gravity and touch. The small number of neurones together with a fixed cell lineage should enable a complete description of the formation of the CNS and its connectivity at a single-cell resolution level (Wada, 1998; Wada *et al.,* 1996, 2006; Bertrand *et al.,* 2003). In the available literatures many authors studied the peripheral nervous system (PNS) of the larvae of ascidians (Torrence and Cloney, 1982; Crowther and Whittaker, 1994; Takamura, 1998; Ohtsuka *et al.,* 2001). The PNS of *Ciona* larvae is composed of a limited number of epidermal sensory neurons (ESN) located in the trunk and tail (Takamura,1998). The tail PNS is made of ESNs (referred to as caudal ESNs or CESNs) scattered at more or less regular intervals along the ventral and dorsal midlines. All CESNs extend along a process in the cellulose-based fin tunic that envelops the larva and are likely to be mechanosensors controlling swimming behavior (Torrence and Cloney, (1982); Crowther and Whittaker, 1994). These cells are described in several ascidian species by Torrence and Cloney, (1982); Takamura, (1998) and Ohtsuka *et al.,* (2001). On the other hand, their developmental history and specification mechanisms are still poorly understood.

2. Materials and Methods

Adult specimens of *Ascidia mentula* (Müller, 1776) were collected from the sub-littoral region of the Mediterranean Sea, Egypt during October, 2008 (breeding season according to Saad, 2008). Identification of this ascidian was carried out according to Millar (1971). The ascidians were transferred into aquaria and maintained at 18-20°C. Artificial heterologous insemination in this species is highly successful, since the genital ducts contain only ripe gametes. This fertilization is carried out in plastic Petri-dishes containing pasteurized and filtered sea water. These may be obtained by slitting open the test and pipetting the eggs and sperm from the oviduct and sperm duct respectively. Fine scissors can be used to puncture the ducts. The eggs remain viable for 18 hours after removal. They should be passed through several changes in sea water before insemination, to free them from the perivisceral fluid. Naturally-spawned eggs should be collected with a small-mouthed pipette and placed in fingerbowls of fresh sea water. The artificially-obtained eggs should be inseminated with a sperm suspension sufficiently concentrated to impart a faint milkiness to the sea water in which the eggs are contained. After fertilization, the essential requirements for normal development are the complete removal of excess sperm and oviduct fluid. Hatching started 18 hr after fertilization, at 18°C. The swimming larvae (6-8 hr after hatching) are withdrawn with a pipette and utilized for optical axiomicroscopy and electron microscopy investigations.

2.1. Microscopic observation

Adult specimens were dissected alive in seawater and the nervous system with a part of the mantle underneath was isolated. The nervous systems was fixed in 10% formalin and washed in distilled water for 24 hours. Dehydration takes place through an ascending series of ethyl alcohol, followed by another dehydration series of tertiary butyl alcohol, then tertiary butanol and paraffin oil (1:1) and finally in pure paraffin oil. All preparations were washed in tissue mate (paraplast) with melting point 54-58 °C and

blocked in fresh paraplast. Sections of 5-8 µm were obtained. Ortholux Leintz Wetzler Stereoscopic microscope with 10, 40 and 100 magnification capacities and Lampenhaus 250 with external light source of Schott KL 1500 was used. The Camera used was full-automatic microscope camera for research and laboratory purposes.

2.2. Scanning electron microscopy (SEM)

The swimming larvae of *Ascidia mentula* were fixed in PAF (picric acid-formaldehyde) 1200 mOsm pH 7.5. The fixed larvae were dehydrated in a graded ethanol series. The dehydrated larvae were critical point dried, mounted on specimen holders, and subsequently sputter-coated with gold. Specimens were examined and photographed using a FEI Quanta 200 SEM at 15 kV.

2.3. Transmission electron microscopy (TEM)

Adult specimens were dissected alive in sea water and the nervous systems with a part of the mantle underneath were isolated. The nervous system was fixed in 2.5% Glutaraldehyde in 0.05 M PBS containing 0.33 M NaCl (1h, 4°C). The fixative was removed by washing specimens several times with PBS. Post-fixation was carried out using 2% Osmium tetroxide in PBS for 30-60 min at 4°C. Specimens were subsequently washed with PBS, dehydrated in a graded ethanol series, and propylene oxide and embedded in araldite resin. Semithin sections and ultrathin (60-70 nm) sections were obtained using Leica UC6 microtome equipped with diamond knives. Ultrathin sections were picked with formvar-coated singleslot copper grids, stained automatically with uranyl acetate and lead citrate in a Nanofilm TEM STAINER, and examined on a Phillips CM 120 transmission electron microscope at 60 kV. Semithin sections were placed on glass slides and stained with toluidine blue (1% toluidine, 1% $Na_2B_4O_7$, 20% sucrose) for 1 min at 60°C.

3. Observations and Results

Trials have been done to prepare histological sections for early larval stages to follow up the different events taking place inside the cerebral ganglion during development. All trials have failed because these larval stages are very delicate tissue measuring about 1.5 – 2 mm. The larvae are very translucent to the extent that the internal organs can be seen and the cerebral vesicle contains pigment granules only. So, live larval stages were stained with borax carmine and studied under Stereomicroscope. Other larval stages were fixed in 2.5% Glutaraldehyde in 0.05 M PBS containing 0.33 M NaCl and used for the description of the development of the larval nervous system.

In *Ascidia mentula*, gastrulation takes place by invagination and epiboly about 7 hrs after fertilization. The nervous system appeared as a neural plate in the late gastrula stage Figure 1. Then invagination of the neural plate proceeds. Closure of the neuropore takes place by fusion of the medullary folds about 11 hr after fertilization Figure 2. Very soon after the commencement of the curvature of the embryo within the follicle, the curvature being initiated and necessitated by the outgrowth of the tail Figure 3. After the first closure of the neuropore has taken place, the nervous system of the embryo consists of a perfectly closed tube lying immediately below the epidermis, and containing a lumen which is slightly dilated anteriorly to form the cerebral vesicle Figures 4 and 5. After 19 hrs of fertilization (hatched larva with long tail), sensory pigments appear inside the brain vesicle and deposited in certain cells of the dorsal wall of the brain vesicle. At this early stage, the statolith is represented by scattered rounded pigment granules lying in several rows of the cells in the dorsal wall of the cerebral vesicle. The otolith and the statolith (the ocellus) appear simultaneously Figures 6 and 7. The granules which belong to the otolith have at first the same characteristics and nearly the same size as those which go to form the statolith. As they increase in number they become very much smaller, and then lie entirely at the inner free extremities of the cells. The otolith granules do not increase in number, but retain their original size until they fuse together. The transparency of larval stages facilitates the present author to follow up the different developmental events of the cerebral vesicle. The migration of otocyst is not an active one, but goes hand in hand with a change in the histological character of the wall of the cerebral vesicle. The ocellus is composed of a cup-shaped pigmented cell, a number of photoreceptors and lens cells Figures 5-8. The observed histological change is correlated with the expansion of the original slight anterior dilatation of the nerve tube into a spacious vesicle. A small portion of the cavity of the cerebral vesicle begins to separate from the main cavity on its left side. A portion of the cerebral vesicle begins to constrict to form of a tube. This tube ends blindly in front, and is communicated behind with the main cavity of the vesicle Figure 8. It lies entirely in the thickness of the wall of the vesicle. By a local thinning out or cuticularization of the wall of the cerebral vesicle, the otocyst (the pigment granules of the otolith) is shifted from its primary dorsal position to its secondary position on the floor of the cerebral vesicle. Meanwhile, the statocyst comes to occupy the posterior right-hand corner of the cerebral vesicle Figure 8 about 130 hr after fertilization (partially metamorphosed larva with short tail, (see Saad and Hamd, 2009). At this stage of development, the tadpole larva is urodele-like in appearance. The trunk has a sensory vesicle with a statolith and an otolith. The neurenteric canal have been obliterated at a somewhat earlier stage. At a later stage, after the commencement of the metamorphosis, a

corresponding opening is actually formed between the cavity of the cerebral vesicle and the stomodaeum Figure 5.

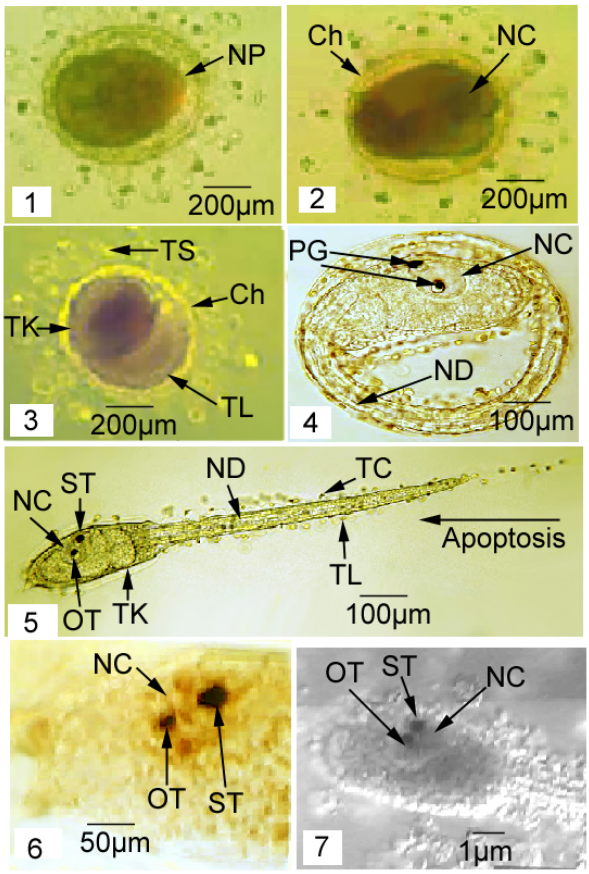

Plate 1

As metamorphosis proceeds, the cerebral vesicle of the larval stage undergoes complete histolytic disintegration and disappears gradually. The neural complex in adult *Ascidia mentula* is about 0.56 mm in animals measuring 7 cm height and 3 cm width and appeared as a mass of tissues which hardly distinguished macroscopically. It has two anterior- two posterior- and one median nerve trunks Figure 9. By using both fluorescent and electron microscopy, this nervous tissue can be differentiated into nerve ganglion and neural gland Figures10-14. A process from the neural gland extends as a cord and terminates at the vicinity of the gonad Figures 15. The ganglion becomes exceptionally large at this particular stage. The nervous system appears solid, but a lumen still persists in front, which opens anteriorly through a funnel-like dilatation, the dorsal tubercle which is a labyrinth-like structure and has possibly arisen in part by evagination from the stomodeum. The nerve cell bodies inside the ganglion are relatively small and seen surrounded a central core of nerve fibers. Some neurons have abundant mitochondria and no granular components while others have numerous dark granules and other neurons still have transparent granules Figures 16-19. The latter two cell types have characteristic features of neurosecretory cells described in other animals Figure 16. Almost all neurosecretory cells in the ganglion apparently belong to the second cell type. Axon bundles containing the same types of granules as in neurosecretory cells were often observed only near the ganglion. The bundles were seen just beneath the epidermis. Other bundles of myelinated and unmyelinated nerve fibers run deeper and do not contain granular components Figures 20-22. In the nerve ganglion, the gap junctional intercellular communication is commonly observed in glial cells. They have many different roles in neural development, though they are typically described as "supportive", and have the same early embryonic origins as neurons and they have many forms Figures 23 and 24.

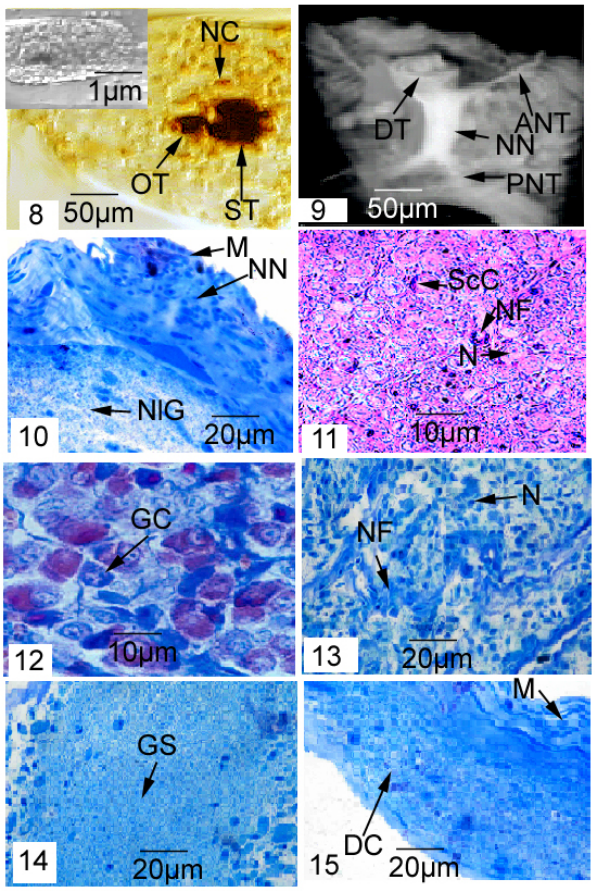

Plate 2

The motor neuron has an axon that extends from the nerve cell body to the Axon's destination. Figure 21 shows a group of large myelinated axons of motor neurons that course through a peripheral nerve. Each axon contains a core of pale axoplasm. The axoplasm contains many slender mitochondria and is supported by cytoskeletal elements including microtubules and neurofilaments. The microtubules and neurofilaments are too small to resolved individually at this magnification and appear as wispy strands. Each of these axons is covered by an electron-dense myelin sheath Figure 25. The myelin sheath is composed of the compressed, spiral wrappings of the Plasma membrane of a Schwann cell which acts as an electrical insulator. The relatively "empty" appearance of the axoplasm is due to the absence of cytoplasmic organelles associated with protein synthesis. The Axon has neither rough endoplasmic reticulum nor free ribosomes.

Consequently, its protein must come from another source, the cell body. All of the nerve fibres within the nerve ganglion are surrounded by a delicate supportive system of loose connective tissue fibrils. This connective tissue is elaborated by a small number of fibroblasts. The neurosecretory neurons occupy a central position in neuroendocrine interactions, not only because it is geared for communication with the endocrine apparatus, but because it serves as a singular channel ("final common path,"). Many secretory granules scattered along the dorsal strand and within numerous neurons of the cerebral ganglion Figures 26 and 27. The cell membrane of the axon is surrounded by a myelin sheath which when examined critically has a laminated appearance. The layers of the myelin sheath consist of tight, spiral wrappings of the plasma membrane of the Schwann cell that surrounds the axon. At the point of synaptic contact between axon and dendrite, the space between adjacent cell membranes is expanded to form the synaptic cleft. A number of synaptic vesicles, filled with neurotransmitter material, are clustered within the axon terminal near the synaptic cleft Figures 26 and 27. Secondary lysosome are observed near the neurosecretory granules inside the nerve ganglion Figures 28 and 29.

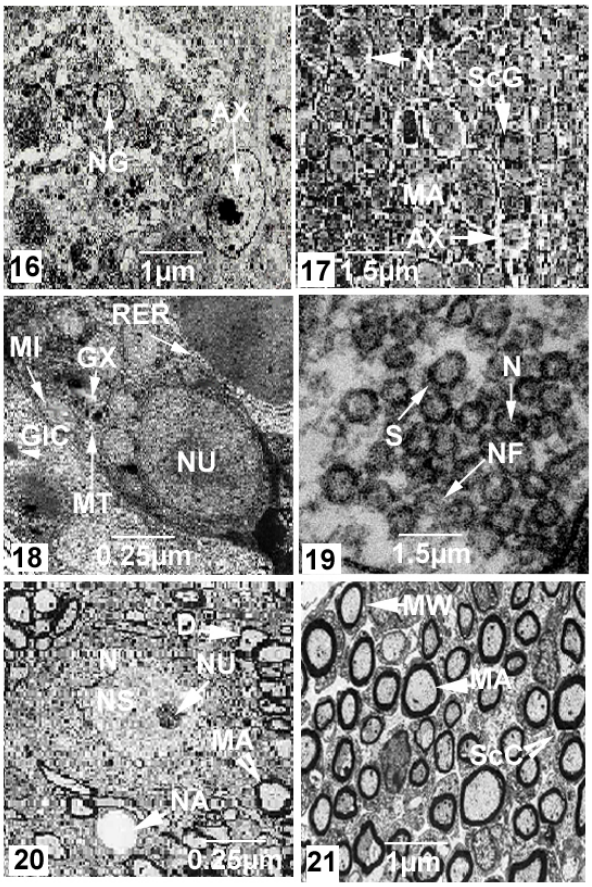

Plate 3

Two different morphological phases were observed in the gland cells: an epithelial phase in which the gland cells are either cubical or irregular in outline comprising mononucleated and binucleated cells Figure 30. Cell proliferation occurs during the epithelial phase.

The glandular laminae are constituted of two layers of prismatic cells which are joined by desmosomes and surrounded by a thin fibrous and sinuous lamella. This lamella stretches when the size of the gland cells increases during the transformation to the mesenchymal phase which is highly vacuolated and lacunated. A new cycle begins when the two layered lamellae are reconstituted forming the remaining cells. These cells contain numerous small vesicles and large vacuoles Figure 31. At the same time, deposited materials were accumulated in the cisternae of the endoplasmic reticulum. Some of the large vacuoles contain an electron dense material or a fibrillar substance. The glandular cells contain no obvious electron opaque secretory granules which are usually associated with an extensive Golgi complex.How ever the vesicles and vacuoles which are certain types of lysosome representing autophagic vacuoles Figure 31. Golgi complex is also found in many vacuoles. Cytoplasmic vacuolation ultimately leads to a breakdown and release of the vacuolar products. The significance of these observations is considered, particularly with respect to the hypothesis that this ascidian gland represents or equivalent of the vertebrate pituitary, gland.

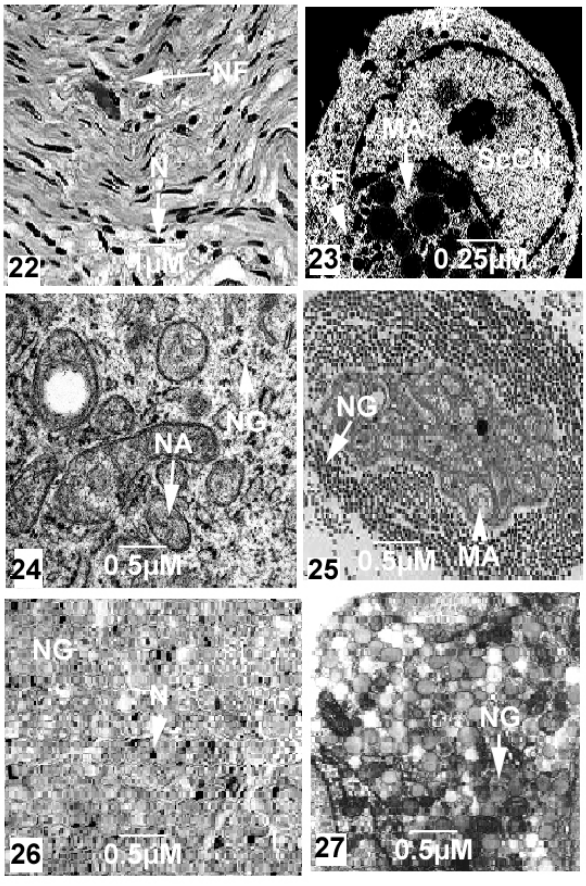

Plate 4

In neurons, cytoplasmic inclusions show some variation in number and size in correspondence with the structural modifications of the gland. When the gland is in the mesenchyme phase, the neurons have a well developed Golgi complex with a number of

saccules and numerous vesicles. These structures seem to aggregate rough endoplasmic reticulum (RER) which sometimes filled with homogenous material. Ribosomes and polysomes are numerous and attached to RER or found free in the cytoplasm between mitochondria Figure 32. During the epithelial phase of the gland, the same structures are found but the Golgi complex is much reduced. Vacuoles and dense bodies are found in different neurons. The nature of these dense bodies may represent a sort of storage of secretory products or be considered as autophagic vacuoles. They might appear whenever too much materials were produced or during the time when the neuron membrane blocks its release. The neurons located at the periphery of the ganglion showed alternation of such vacuoles and dense bodies which appeared to be secondary lysosomes. This alternation seems to be synchronized with the cycle shown by the neural gland. This finding leads us to suppose that the nerve ganglion controls the cycle of the gland cell morphology. Because of the anatomical relationship of the gland with the pharynx, the neural complex have been homologated to the adenohypophysis.

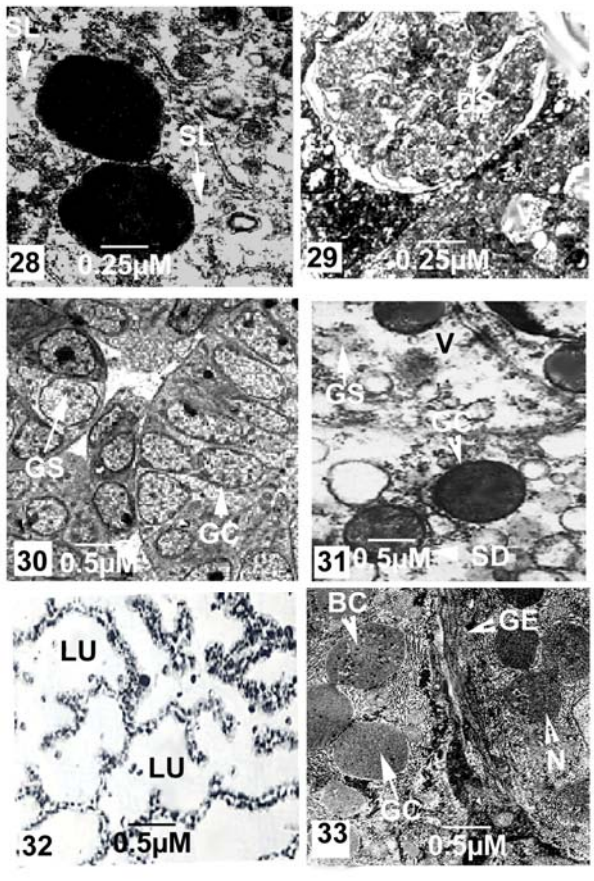

Plate 5

The fibrous lamina associated with the membranes around the glandular portion of the nervous system is possibly function in transferring the neurosecretory granules from the axonic endings of the ganglion to the neural gland. The dorsal strand, or dorsal cord, is a tubular epithelial structure originating from the posterior end of the neural gland and running back within the dorsal blood sinus Figure 15. This strand extends as far as the gonads, which appear to be formed in association with it during development.

The gland cells are enclosed and bounded externally by an epithelial membrane. This membrane showed three morphological appearance in the different examined histological and transmission electron preparations. Sometimes, it consists of flattened cells, or of columnar cells or from pseudostratified cells Figure 33. In any case these cells are deeply stained and granulated.

4. Discussion

This study reveals that the neural complex of *Ascidia mentula* develops from the neural plate. After the first closure of the neuropore has taken place, the nervous system of the embryo consists of a perfectly closed tube lying immediately below the epidermis, and containing a lumen which is slightly dilated anteriorly to form the cerebral vesicle. The sensory pigments, inside this vesicle, begins to appear after 19 hrs of fertilization. These pigmented granules are deposited in the interior of certain cells of the dorsal wall of the cerebral vesicle. Nicol and Meinertzhagen (1988) reported that the ascidian neural plate differs from its amphibian homologue by having fewer blastomeres and by undergoing relatively more cleavages and neurulation before sinking beneath the ectoderm. In contrast to vertebrates, however, the anterior most region of the neural plate does not roll up and internalize but contributes to the so-called dorso-anterior epidermis, which includes the adhesive organs, head sensory neurons, and pharynx (Nishida, 1987; Bertrand *et al*., 2004 ; Brown *et al*., 2004). The neural plate gives rise to three structural divisions of the larval ascidian nervous system (Bone and Mackie, 1982). These divisions are:- (a) a rostral sensory vesicle containing the sensory receptor systems, with a pigmented ocellus (Dilly, 1964) and, anterior to it, a pigmented otolith (Dilly, 1962); (b) a caudal nerve cord and (c) a visceral ganglion containing the motor neurons (Nicol and Meinertzhagen, 1991; Meinertzhagen *et al.*, 2004). The cerebral vesicle of ascidian larvae, in general contains two distinct pigmented sensory organs clearly visible through its transparent body. The more anterior pigmented sensory organ, the statolith, is involved in the perception of gravity, whereas the more posterior one the ocellus, is involved in the perception of light stimuli. The two sensory organs are responsible for the swimming behaviour of the larva (Tsuda *et al.*, 2003). Live materials stained with borax carmine showed cup-shaped pigmented cells of the ocellus, a number of photoreceptors and three lens cells. These observations were previously reported by Dilly (1969) on *Ciona intestinalis*, *Phallusia mammilata* and *Ascidia nigra* and by Nicol and Meinertzhagen (1991) and Meinertzhagen, *et al.* (2004) on *Ciona intestinalis*.

The nervous system of a hatched larval stage of *Ciona intestinalis*, *Phallusia mammilata* and *Ascidiella aspersa* is represented by a cerebral vesicle in the trunk region containing the statolith and an ocellus and a long neural tube having a central canal in the tail region (Saad, 2002; Hofmann *et al.* 2008; Michael *et al.* 2008; Saad and Hamed, 2009). This study added that the process of metamorphosis of the larval stage together with the apoptosis which occurs in caudal region of the larval stage are concomitant with the degeneration of the neural tube and autolysis of both statolith and the ocellus in the cerebral vesicle. Rotation of the internal viscera bring the nervous system of the newly metamorphosed animal to lie in between the branchial and the atrial siphons. The neural complex is now represented by a nervous mass of tissues completely embedded in the mantle epithelium and hardly to be differentiated into components macroscopically. Fluorescent and electron microscopy examination show that the neural complex can be differentiated into nerve ganglion, neural gland and a dorsal tubercle. A dorsal cord extends from the neural gland and terminates at the vicinity of the gonad. The neural gland opens at the apex of the branchial chamber through a ciliated funnel carried on a dorsal tubercle. In the current study, the motor nervous system of adult *Ascidia mentula* consists of the cerebral ganglion with axons run out directly to the effectors. The nerve cell bodies inside the ganglion are relatively small and surround a central core of mylinated and non-mylinated nerve fibers. These fibers could be classified ultrastructurally into three types by their cytoplasmic contents: cells having abundant mitochondria and no granular components, cells having numerous dark granules and cells having transparent granules. It can be speculated that these membranous organelles perform two functions inside the neural complex. Firstly, they are responsible for the intracellular digestion of macromolecules. A wide variety of enzymes that break down macromolecules such as nucleic acids, proteins, and polysaccharides are present inside these organelles. Secondly, they breakdown old nonfunctioning organelles that out lived their usefulness. These lysosomes are needed to rid the cell of these unneeded materials that are occupying spaces in the cell. The lysosomal enzymes digest all organelles and may speed up the cells' death by this process of autolysis (self-digestion)

Georges (1971) and (1978) showed that the ventral region of the nerve ganglion of newly metamorphosed *Ciona intestinalis* generates oligodendrocyte precursors initially which then migrate both laterally and dorsally. In a late stage, the dorsal region provides a secondary source of oligodendrocyte precursors. Microglia are distributed randomly inside the nerve ganglion. Glial lineage is differentiated from the neural cells, which in turn generate Schwann cell precursors. Nicol and Meinertzhagen (1991) showed that the axons grow through the extracellular matrix and reach their targets from different directions. In the sensory vesicle, the nerve cells are numerous, especially in the posterior wall of the vesicle (Cole and Meinertzhagen, 2004). Adult ascidian brain differs from that of larvae themselves, with a considerable variation in the statolith and otolith of the sensory vesicle (Vorontsova, 1988 ; Gilbert,and Raunio, 1997). Cell numbers in urochordate brains is generally small, but their numbers of other urochordate groups differ somewhat from those in ascidian larvae. In salps, the numbers of cells are not reduced as in the symmetrical dorsal ganglion, which is thus more easily compared with the more complex brains of chordates (Lacalli, and Holland, 1998 ; Lemaire *et al.,* 2002). Analysis of the pattern of nervous system embryogenesis of the deuterostome, as the hemichordate *Saccoglossus kowalevskii*, suggests that it is a chordate nervous system (Lowe, 2008).

This present study deals with the investigation of the cerebral ganglion and its main nerves and the neural gland of the adult stage since the investigation of the peripheral nerves is very difficult to continue. It needs other techniques and other possibilities. However the peripheral nervous system (PNS) of ascidians was the interest of other authors (Jia, 1987 ; Nishida, 1987 ; Takamura, 1998 ; Crowther and Whittaker, 1994). Ascidian larvae have a system of 30–40 epithelial neurons in the tail (dorso- and ventro-caudal epidermal neurons) and the head, or trunk (apical- and rostral-trunk epidermal neurons), which form a simple PNS (Takamura, 1998). At least some of these peripheral neurons are likely mechanoreceptors. They are embedded in the epithelium (Jia, 1987), extend long cilia into the tunic of the tail (Crowther, and Whittaker, 1994), and are connected to nerves that run back to the sensory vesicle. In neither *Ciona* nor *Halocynthia* does the tail epidermal neurons derive from the neural plate but rather from the midline epidermal territories (Nishida, 1987). This origin of the tail sensory neurons expresses a neural crest–like genetic program which is not the case for the ventral sensory neurons.

For the neuromuscular apparatus, in particular, Bone (1992) has spoken against considering the ascidian tadpole larva as a chordate prototype. For the larval nervous system, too, there are major differences. The larval brain lacks clear signs of segmentation and laminae and retains the character of a hollow epithelial tube, whereas chordate brains, especially craniate, undergo lamination by radial migration. Given this difference, the fact that *Ciona* has the gene reelin, which in mammals plays a role in organizing the brain's layers (Gilbert and Raunio, 1997 ; Curran *et al.,* 2003). Associated with the lack of lamination is the lack of stem cells in the developing brain of ascidian embryos, in which cells arise through equal cleavages, at least as so far identified. The relative absence of radial cell migration and massive cell death distinguish this simple brain from its more complex vertebrate counterpart. Likewise, the absence of myelinated nerves and axons in both fiber tracts of the CNS (Katz, 1983) and peripheral nerves (Torrence, 1983), confirmed by the absence of myelin-related genes in the *Ciona* genome (Dehal, *et al.*, 2002),

bespeaks a lack of rapid conduction pathways and a simpler, possibly ancestral chordate organization. The latter possibly correlates with, in the case of myelination genes, (*a*) the lack of neuregulins, which are involved in axon-oligodendrocyte signaling (Canoll *et al.,* 1996), and of orthologues for the oligodendrocyte determinants Olig1 and 2; and (*b*) the lack of neurotrophins and their receptors (Dehal *et al.,* 2002), the presence of which promotes survival and neuron extensions.

According to the neural gland in ascidians, the present study showed two different morphological appearances in the gland cell morphology: an epithelial phase and a mesenchymal phase. Georges (1971) revealed that this cyclical change in the gland cell morphology in *Ciona intestinalis* depends on the tide and has no relation with the breeding season. The gland cells become increasingly vacuolated as their shape decreases in regularity. At the same time, depositions accumulate in the cisternae of the endoplasmic reticulum. Some of the vacuoles contain an electron dense material or a fibrillar substance, but the cells contain no obvious electron opaque secretory granules associated with an extensive Golgi complex. These vesicles and vacuoles are possibly representing autophagic vacuoles. Golgi complex is also found in many vacuoles. The results suggest a developmental cycle of increasing cytoplasmic vacuolation, ultimately leading to a breakdown and release of the vacuolar products. The significance of these observations is considered, particularly with respect to the hypothesis that the gland represents the ascidian equivalent of the vertebrate pituitary (Deyts *et al.,* 2006).

Georges (1971) studied the neural gland of *Ciona intestinalis* and revealed that this gland is comparable to vertebrate adenohypophyses. The nature of the dense bodies inside the neurons may represent a sort of storage of secretory product or be considered as autophagic vacuoles. They could play a role in regulating the secretory process. They might appear whenever too much material was produced or during the time when the neuron membrane blocks its release. This material might carry the message responsible for the cycling of the gland. The neurons located at the periphery of the ganglion showed alternation of much vacuoles and dense bodies which appeared to be secondary lysosomes. This alternation seems to be synchronized with the cycle shown by the neural gland. This finding showed that the nerve ganglion controls the cycle of the gland cell morphology.

The fibrous lamina associated with the membranes around the glandular portion of the nervous system possibly function in transferring the neurosecretory granules from the axonic endings of the ganglion to the neural gland. The dorsal strand, or dorsal cord, is a tubular epithelial structure originating from the posterior end of the neural gland and running back within the dorsal blood sinus. This strand extends as far as the gonads, which appear to participate in gonadal development.

The gland cells are enclosed and bounded externally by an epithelial membrane. This membrane showed three different morphological epithelial cells; such as of flattened cells, and /or pseudostratified cells . Earlier investigators attributed various functions to the gland: It acts as an excretory system (Millar, 1953) or as a mucus gland (Roule, 1884). In addition, it acts as an endocrine organ as reported by Carlisle (1951) and Dodd (1955). These studies have resulted in much disagreement, and the function and structure of the gland in ascidians remain controversial. Neurosecretory cells have been shown to occur in the neural ganglion of ascidians, one of the main components of the neural complex in this animal (Dawson, and Hisaw, 1964; Lane *et al.,* 2001).

According to some authors the ascidian ganglion exhibited a gonadotropic function (Hisaw *et al.,* 1966 ; Georges, 1971; Bouchard-Madrelle, 1967 ; Lacalli and Holand 1998). An acceptable interpretation of function (s) of the neural ganglion and gland has not been proposed. Many recent studies on the neurosecretory phenomena in various invertebrates other than ascidians have been carried out (Tombes *et al.,* 1992; Golding and Dean, 1998). Accumulating evidence from such studies indicates that the neurosecretory system in invertebrates plays an important role in reproductive activity. Parallel with this result, the neurosecretory system may serve a similar function in ascidians. As an approach to this subject, the functional criteria for neurosecretory status suggested by Bern (1962) were applied and a possible role for the neural complex in reproductive activity in *Symplegma reptans* was postulated. The cells comprising the neural gland in the ascidians *Ciona, Styela*, and *Botryllus* have been examined for their fine structural features and enzyme cytochemistry. However, Bern found accumulation of glycogen deposits and the distended cisternae of the endoplasmic reticulum. Some vacuoles contain an electron dense material or a fibrillar substance, but the cells contain no obvious electron opaque secretory granules associated with Golgi complex similar to that found in the vertebrate adenohypophysis. Acid phosphatase is localized in some of the vesicles and vacuoles, indicating that they are a kind of lysosomes, representing autophagic vacuoles. Thiamine pyrophosphatase is also found in many vacuoles as well as in the saccules of the Golgi apparatus which is represented by of dictyosomes. The results of Manni *et al.* (2004) suggested a developmental cycle of increasing cytoplasmic vacuolation, ultimately leading to a breakdown and release of the vacuolar products. The significance of these observations is considered, particularly with respect to the hypothesis that the gland represents the ascidian equivalent of the vertebrate pituitary. This study was in agreement with the suggestion of Bern (1962); Georges (1971) and Terakado (2009) that the neural gland in *Ascidia menula* is comparable to the vertebrate pituitary and disagree with the suggestion of Manni *et al.* (2004) and Lane *et al.* (2001). In addition, Terakado (2009)

reported that gonadotropin-releasing hormone is produced from the neural complex of *Halocynthia roretzi*. Furthermore, adrenocorticotropin-like immunoreactivity in the granules of the neural complex cells of the ascidian *Halocynthia roretzi* were previously identified (Kawahara *et al.*, 2002).

List of abbreviations

ANT Anterior Nerve Trunk
AP Axioplasm
AX Axon
BC Binucleated Cell
Ch Chorion
D Dendrites
DC Dorsal Cord
DS Degenerated Cells
DT Dorsal Tubercle
GC Gland Cell
GE Gland Epithelium
GlC Glial Cells
GS Gland Secretion
GX Golgi Complex
LU Lacunae
M Mantle
MA Myelinated Axons
Mi Mitochondria
MT Microtubules
MW Mylin wrapping over Axon
N Nerve Cell Body
NA Non-Mylinated Axon
NC Neural Complex
ND Nerve cord
NF Nerve fibre
NG Neurosecretory Granules
NlG Neural Gland
NN Nerve Ganglion
NP Neural Plate
NS Nucleus
NU Nucleolus
OT Otolith
PG Pigment Granules
PNT Posterior Nerve Trunk
RER Rough Endoplasmic Reticulum
S Synaptic Endings of other Neurons
ScC Schwann Cell
ScCN Schwann Cell Neucleus
SL Secondary Lysosomes
ST Statolith
TC Test Cell
TK Trunk
TL Tail
TS Test Cell
V Vacuoles

Explanations of Figures

Figure 1: Phase contrast photomicrograph of a late gastrula of *Ascidia mentula* showing the flattening of the ectoderm from the ventral side to form the neural plate (NP).

Figure 2: Phase contrast photomicrograph of a late neurula stage of *Ascidia mentula*.

Figure 3: Phase contrast photomicrograph of tail bud stage of *Ascidia mentula*.

Figure 4: Phase contrast photomicrograph of a fully-formed larval stage of Ascidia mentula inside the chorion.

Figure 5: Phase contrast photomicrograph of a hatched larval stage of *Ascidia mentula*.

Figure 6: Phase contrast photomicrograph of an enlarged trunk region of a hatched larval stage of *Ascidia mentula* showing the neural complex.

Figure 7: Scanning electron micrograph of the trunk region of a hatched larval stage of *Ascidia mentula* showing the neural complex.

Figure 8: Phase contrast photomicrograph and SEM for the same trunk region (found on the upper left side) of a whole mount of an enlarged trunk region of a partially metamorphosed larval stage of *Ascidia mentula* showing the neural complex

Figure 9: Phase contrast photomacrograph of a dissected neural complex of a young stage of *Ascidia mentula.*

Figure 10: Semithin section through the neural complex of adult stage of *Ascidia mentula.*

Figure 11: Photomicrograph of a transverse section through the neural complex of *Ascidia mentula* showing the nerve ganglion.

Figure 12: Photomicrograph of a transverse section through the neural complex of *Ascidia mentula* showing the neural gland.

Figure 13: Semithin section through the neural complex of *Ascidia mentula*
showing the nerve ganglion.

Figure 14: Semithin section through the neural complex of *Ascidia mentula*
showing the neural gland.

Figure 15: Semithin section of *Ascidia mentula* showing the dorsal strand of the neural gland.

Figure 16: Transmission electron micrograph of a transverse section of the neural complex of *Ascidia mentula* just under the level of the two anterior nerve trunks showing neurosecretory granules of different sizes.

Figure 17: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex of *Ascidia mentula* showing nerve cell bodies surrounded by nerve processes. The neuronal surface is completely covered by either synaptic endings of other neurons or processes of glial cells.

Figure 18: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex of *Ascidia mentula* showing cell body of neurons at higher magnification. An evident nucleulus (NU) presents in the nucleus. In the cytoplasm numerous mitochondria (Mi), are seen, a Golgi complex (GX) and rare profile of

rough endoplasmic reticulum vesicles (RER). Inside the axon are present and bundles of microtubules running parallel to the longitudinal axis. Neurons have electron-transparent granules.

Figure 19: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex showing the nerve ganglion about the middle region of the neural complex. Electron-dense granules are seen in the neuron. Nerve cell bodies (N) and nerve fibres (NF).

Figure 20: Transmission electron micrograph of a transverse section of *Ascidia mentula* showing a single nerve cell body (N) surrounded by nerve processes.

Figure 21: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing myelinated axons (MA).

Figure 22: Transmission electron micrograph of a longitudinal section through the neural complex of *Ascidia mentula* showing non-myelinated axons (NA).

Figure 23: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex showing a Schwann cell (ScC) resting on a myelinated axon (MA).

Figure 24: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the glandular nature of the dorsal cord.

Figure 25: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex showing the posterior nerve trunk.

Figure 26: Transmission electron micrograph of a transverse section of *Ascidia mentula* through the neural complex showing the nerve ganglion and neurosecretions.

Figure 27: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the dorsal strand and neurosecretory granules (NG).

Figure 28: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the nerve ganglion and secondary lysosomes (SL) near the neurosecretory granules (NG).

Figure 29: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the neural gland. Degeneration of epithelial cells to form its holocrine secretion and secondary lysosomes (SL).

Figure 30: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the neural gland in its epithelial phase.

Figure 31: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the gland cells vacuolation.

Figure 32: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the neural gland in its the mesenchymal phase.

Figure 33: Transmission electron micrograph of a transverse section through the neural complex of *Ascidia mentula* showing the pseudostratified epithelial wall of the neural gland.

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دراسات مجهرية وميضية و تركيبية دقيقة لعملية نمو الجهاز العصبي في أسيديا منتولا (مولر ، 1776). ذيلحبليات - غلاليات

جابر أحمد سعد

قسم علم الحيوان- كلية العلوم - جامعة الأسكندرية

جمعت عينات الأسيديا منتولا من المناطق الساحلية للبحر الأبيض المتوسط في سبتمبر 2008.

تم عمل تلقيح أصطناعي مختلط في المعمل و الحصول علي المراحل الجنينية المختلفة اللازمة للدراسة.

ظهر الجهاز العصبي علي هيئة صفيحة عصبية - أكتودرم عصبي - في مرحلة متأخرة من الجاسترولا ، ثم حدث إنغماد تدريجي للصفيحة العصبية لتكون بذلك الأنبوبة العصبية. وبمرور الوقت يتسع تدريجيا الجزء الأمامي من الأنبوبة العصبية مكونا الحويصلة الدماغية. و مع زيادة النمو تترسب حبيبات صبغية في المناطق الداخلية لخلايا معينة مكونة الجدار الظهري للحويصلة الدماغية.

و تظهرالعين البسيطة علي هيئة حبيبات صبغية صغيرة و متناثرة في عدة صفوف داخل الخلايا المكونة للجدار الظهري للحويصلة الدماغية. ويظهر تجمع آخر من الحبيبات الصبغية يمثل عضو الإتزان والذي يظهر بجانب العين البسيطة. و تظهر الحبيبات التي تنتمي إلي العين البسيطة في البداية بنفس الخصائص وتقريبا بنفس حجم تلك التي تميل إلى تشكيل عضو الإتزان وتكون منتشرة في جميع أنحاء الخلايا و تدريجيا تزداد أعداد هذه الحبيبات و يقل حجمها كثيرا و تتركز على الحدود الداخلية للخلايا. أما الحبيبات التي ستكون عضو الإتزان فإنها لا تميل إلى الزيادة في العدد ، ولكن تحتفظ بحجمها الأصلي حتى تلتحم مع بعضها.

يتكون الجهاز العصبي في مرحلة اليرقات حديثة الفقس من أنبوب عصبي مغلق تماما و يقع مباشرة أسفل البشرة ، ويحتوي على تجويف مركزي. و يظهر الجهاز العصبي في الطور حديث التحول علي هيئة كتلة عصبية لها جذعين عصبيين أماميين و جذعين عصبيين خلفيين و جذع عصبي أوسط.

ويظهر الميكروسكوب الفلورسنتي المجهري والألكتروني النافذ أن هذه الكتلة العصبية تتكون من عقدة عصبية ، وغدة عصبية و مجرى عصبي. يمتد الحبل الظهري من الغدة العصبية وينتهي فوق الجهاز التناسلى مباشرة. وتظهر أجسام الخلايا العصبية داخل العقدة العصبية صغيرة نسبيا ، وتحيط بكتلة مركزية من الألياف العصبية الميلينية و غير الميلينية. و يوجد ثلاث أنواع من الخلايا العصبية : خلايا تحتوي علي كميات وفيرة من الميتوكوندريا ولا تحتوي علي حبيبات و خلايا آخري تحتوي علي حبيبات كثيفة و آخري تحتوي علي حبيبات رائقة.

لوحظ داخل العقدة العصبية خلايا غرائية متعددة الآشكال ، حبيبات إفرازية عصبية متعددة ، أجسام جولجى ، شبكة إندوبلازمية ماكنة ، ميتوكوندريا و أجسام من الريبوسومات الثانوية. و تتكون الجذوع العصبية من آلياف ميلينية و غير ميلينية. كما تظهر الغدة العصبية خلوية و تحتوي علي خلايا طلائية ذات

نواة أو نواتين كما أنها تظهر أيضا علي شكل فجوي وتحتوي علي خلايا متحللة و إفرازات غدية. ويتكون النسيج الطلائى المبطن للغدة من خلايا حرشفية أو خلايا مكعبة أو من نسيج طبقي كاذب.