Development of the nervous system of Aplysia californica
(neurobiology/neuronal development/morphology)

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ABSTRACT The ability to grow the marine mollusc Aplysia under laboratory conditions allows a detailed study of the formation of the nervous system and of the development of specific identified cells. I have found that the ganglia develop in a specific temporal order. Cerebral and pedal ganglia develop at hatching, the abdominal, pleural, and opisthobranch ganglia 3 weeks after hatching, and the buccal ganglia at 4 weeks. The origin of the abdominal ganglion is complex; its anlage forms at 3 weeks from a larval ganglion that fuse to form the abdominal ganglion. Individual cells cannot be distinguished from one another by their location within the ganglion or by their appearance alone until metamorphosis at 5 weeks. After metamorphosis, the identified neuron, R_2, suddenly becomes recognizable because of a significant increase in its size.

The large identifiable neurons that have made the adult (postmetamorphic) opisthobranch mollusc Aplysia californica useful for neurobiology also make premetamorphic Aplysia a valuable experimental animal for developmental studies of the nervous system. For example, the egg of Aplysia is determinate, so that it is theoretically possible to trace specific identified neurons of the adult to their cells of origin in the blastula. Moreover, the diversity of identified neurons in a single ganglion of the adult (neurosecretory cells, sensory cells, interneurons, and motor cells) enables Aplysia to become a potentially useful animal for studying the program for differentiation of different types of neurons. Since synaptic connections have been mapped between identified cells in the adult, the formation of synapses between cells could also be studied.

As a first step in the study of neuronal development in Aplysia, I have described the temporal sequence of formation of the major ganglia. Particular emphasis has been placed on the formation of the abdominal ganglion because it has been well studied in the adult, and because it develops in stages.

METHODS

There are five major phases in the life cycle of Aplysia: (1) the embryonic phase (10 days), (2) the premetamorphic phase (35 days), (3) the metamorphic phase (2 days), (4) the juvenile phase (approximately 85 days), and (5) the adult phase. The four postembryonic phases can, in turn, be subdivided into 13 stages (1).

The structure of the developing nervous system was defined at each stage from serial sections of histological specimens of animals reared in the laboratory (1, 2).

Animals were first narcotized by the propylene oxide method of Leibowitz (3) and then fixed at room temperature for 1 hr in 2.6% glutaraldehyde (20 ml of seawater, 10 ml of 8% glutaraldehyde, 8 drops of 1 M HCl, 3 drops of s-collidine). They were rinsed for 15 min by three changes of s-collidine buffer (20 ml of seawater, 8 drops of 1 M HCl, 3 drops of s-collidine) and post-fixed for 1 hr in 1% osmium tetroxide (10 ml of 2% OsO_4, 10 ml of 0.1 M Sorensen's phosphate buffer, 5.5 g of sucrose). The tissue was dehydrated in alcohol and embedded in Epon. Sections (1–2 µm) were cut on a Porter-Blum LK 2 Ultramicrotome, and stained by the polychrome method of Mackay and Mead (28th Electron Microscopy Society of America Meetings, 1970). Photomicrographs were made of alternate sections, and the shapes of the major ganglia were copied onto tracing paper. The three-dimensional shapes of the major ganglia were determined from these drawings.

RESULTS

Development of the central nervous system

The central nervous system of the adult Aplysia consists of four pairs of head ganglia, the cerebral, pedal, pleural, and buccal, in addition to a single abdominal ganglion. The first ganglia to form are the paired cerebral and pedal ganglia. These are already present during the late stages of embryonic development (within ten days of fertilization) (4). The cerebral ganglia lie on either side above the esophagus and are connected by a broad commissure. Each pedal ganglion lies at the base of the foot and develops together with the otocyst, an organ of position sense (Fig. 1A).

After hatching, Aplysia larvae swim using cilia and feed on unicellular algae. Approximately 14 days after hatching, cells derived from ectoderm overlying the cerebral ganglia develop into eyes. The eyes are linked to the cerebral ganglia by two small clusters of cells—the paired optic ganglia—which lie at the anterodorsal surface of the cerebral ganglion. At this stage the eyes make direct contact with the ganglia since there are no optic nerves.

About 21 days after hatching (and 14 days before metamorphosis) the opisthobranch, abdominal, and pleural ganglia begin to develop. The opisthobranch ganglion develops below the ectoderm lining the superior border of the entrance into the mantle cavity. The ganglion is oblong, lying transversely above the mantle opening on the right side and is linked to the right abdominal ganglion inferiorly.

The pleural ganglia develop along the postolateral surfaces of the cerebral ganglia. Pleuro-pedal connectives pass in front of each otocyst, where they merge with the cerebro-pedal connectives to enter the ipsilateral pedal ganglion (Fig. 1A).

Approximately 32 days after hatching, the buccal ganglia develop on the postolateral surface of the benthic juvenile, approximately 35 days after hatching (2). During the postmetamorphic phase of the life cycle, all the ganglia increase in cell number, the connectives between ganglia lengthen, and some small, peripherally located ganglia develop.

Development of the abdominal ganglion

A detailed example of the way the nervous system develops in stages can be seen in the formation of the abdominal ganglion.

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The abdominal ganglion arises as a fusion of three larval ganglia. The right hemi-abdominal ganglion, which appears to be the homologue of the suprainterstitial ganglion of more primitive opisthobranchs, develops above the esophagus. The left hemi-abdominal ganglion develops below the esophagus and is divisible into a rostral and a caudal part by the appearance of two clusters of neurons, each surrounding a central medulla of neuropile. The rostral cluster, which appears to be the homologue of the subintestinal ganglion, will become the lower left quadrant of the adult abdominal ganglion. The caudal cluster (the homologue of the visceral ganglion) will become the left upper quadrant of the adult ganglion. The commissure connecting the right and left halves of the abdominal ganglion passes from the supra-esophageal ganglion inferiorly around the right margin of the esophagus to enter the subesophageal visceral complex (Fig. 1A and C).

Connectives link the right and left abdominal ganglia to the right and left pleural ganglia (Fig. 1B). During the 7 days prior to metamorphosis, the left hemi-abdominal ganglion consolidates into a single cell cluster slightly larger than the right hemi-abdominal ganglion. After metamorphosis the abdominal commissure becomes shorter and the left abdominal ganglion moves from below to above the esophagus.

Approximately 35 days after metamorphosis, a small cluster of cells, called bag cells by Frazier et al. (5), appears on the dorsal surface of each pleuro-abdominal connective approximately 50 μm from the junction between the connectives and the abdominal ganglion. The bag cells increase in number until each cluster contains about 400 in the adult (6). These two clusters are the last components of the abdominal ganglion to develop.

Appearance of identified neurons R2 and P1

Not only does the abdominal ganglion develop in stages, but different identified cells become morphologically recognizable at different times. The two cholinergic cells R2 and P1, the largest cells of the abdominal and pleural ganglia, can be identified first. Prior to metamorphosis, the right abdominal ganglion contains approximately 100 cells that are 7–10 μm in diameter and cannot be readily identified. In the days following metamorphosis a single cell becomes twice as large as the others. This cell is approximately 20 μm in diameter and is identifiable as R2 by its size and position 7 days after metamorphosis (Fig. 2). At the same time, a cell of similar size appears in the left pleural ganglion and can be identified as cell P1 10 days after metamorphosis. The increase in size of these two cells coincides with the development to the parapodia, the structure to which the two cells send their major axonal branches (8).

DISCUSSION

Development of the Nervous System. An important feature of the development of the nervous system is that the major ganglia do not appear all at once, but at fixed intervals over an extended period. While the cerebral and pedal ganglia form 2 days before hatching, the buccal ganglia do not appear until
just before metamorphosis, 32 days after hatching. It should be possible to exploit the sequential appearance of new ganglia, and perhaps new cells, to analyze the contribution each new component makes to the behavior of the animal and to the structure of the preexisting nervous system.

Development of the Abdominal Ganglion. The nervous systems of primitive opisthobranchs such as Acteon contain five ganglia in the visceral region—a pair of pallial ganglia, a visceral ganglion, and a supra- and subintestinal ganglion. This led Guiart (9) to postulate that the abdominal ganglion of Aplysia represents a fusion of these five ganglia (9). In this paper I describe the formation of the abdominal ganglion of Aplysia by the sequential fusion of three larval ganglia. Based on their positions along the visceral loop, the right hemi-abdominal ganglion and the rostral and caudal parts of the left hemi-abdominal ganglion of the larval Aplysia probably correspond...
to the supraintestinal, subintestinal, and visceral ganglion, respectively, of the archetypal mollusc.

There are at least three explanations for the fate of the pallial ganglia in *Aplysia*. First, they may correspond to the bag cell clusters as originally postulated by Guiart. Second, pallial ganglia analogues may not exist in *Aplysia*. Third, they may be incorporated into other ganglia. Hughes and Tauc (8) found that two large neurons, one in the left pleural ganglion (P1) and one in the right hemi-abdominal ganglion (R2), had a symmetrical distribution of their axons. They therefore postulated that the right pallial ganglion, presumably containing cell R2, fused with the right abdominal complex whereas the left pallial ganglion, presumably containing cell P1, fused with the left pleural ganglion. It may be possible to decide among these possibilities by examining the cells in the pallial ganglia of one of the more primitive living species of opisthobranch. The presence of homogeneous neurosecretory cells resembling those in the bag cell clusters of *Aplysia* would support Guiart's prediction, whereas the discovery of giant cells resembling R2 and P1 would confirm Hughes' and Tauc's notion.

**Development of Identified Cells.** Juveniles are large enough to be handled by dissection techniques 4 days after metamorphosis, and some of the neurons (15 μm and larger) have been studied successfully with intracellular microelectrodes. If the identified neurons are not fully differentiated at this stage, studies of these cells may reveal late stages in the formation of their characteristic electrophysiological and pharmacological properties.

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