J. exp. Biol. (1981), 95, 205–214
With 8 figures
Printed in Great Britain

THE ROLE OF SUPPORT CELLS IN THE GROWTH AND DIFFERENTIATION OF NEURONES IN THE ABDOMINAL GANGLION OF *APLYSIA*

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SUMMARY

During the late premetamorphic stages of development, the abdominal ganglion of *Aplysia* is surrounded by a group of support cells which later develop morphological properties characteristic of glial cells. These support cells contain large secretory granules whose contents are released primarily after the onset of the metamorphic phase. The release of the granule contents may signal the burst of neuronal growth and maturation that occurs following metamorphosis. The evidence supporting this idea is the following: (1) The release of the granule material after the onset of metamorphosis coincides with an increase in cell body growth and a more marked increase in the density of synapses within the neuropil. Both release and neuronal maturation can be blocked when metamorphosis is postponed by withholding the appropriate macroalgal substrate. (2) Premature release of the granule contents 2–3 weeks before metamorphosis with artificial sea water containing a high concentration of potassium results in an increase in cell body growth, density of synapses, and the number of spines formed and contacts received by specific identified cells. (3) Artificially inducing the release of the granule material in animals whose metamorphosis has been prevented (by withholding the appropriate substrate) still produces an increase in cell body growth and density of synapses. These results suggest that the release of material from support cell granules provides a general stimulus for neuronal differentiation including cell body growth, spine development, and synapse formation.

INTRODUCTION

Mature cells in multicellular organisms express specific phenotypes as a direct consequence of the activation of particular classes of genes (Davidson, 1976). The process of development can be defined as the mechanisms by which specific sets of genes are activated and deactivated in different cell groups leading to their acquisition of particular properties. What are the signals for differential gene activity? By analogy with work on gene induction in both prokaryotes and eukaryotes (Brown, 1981), it is now generally believed that environmental or epigenetic signals can modulate gene expression during development. Thus, the differentiation by neurones of unique cellular properties and interconnexions proceeds via a developmental sequence which includes temporally and spatially specific epigenetic cues whereby certain portions of

the cell's genome (neurone–specific) is selectively activated and modulated (Stent 1981). The sources of epigenetic signals affecting neuronal development can be classified into two categories: (1) the local environment and (2) the external environment. The local environment includes various examples of cell surface interactions which could aid neurones in (*a*) migration to appropriate positions (Jacobson, 1978; Varon & Somjen, 1979); (*b*) growing processes towards the correct target (Lopresti, Macagno & Leventhal, 1973; Letourneau, 1975; Caviness & Rakic, 1978; Silver & Sidman, 1980); and (*c*) recognizing the correct target for synapse formation (Sperry, 1963; Moscona & Hausman, 1977; Jacobson, 1978; Rutishauser *et al.* 1978; Gottlieb & Glaser, 1980). In addition, the diffusion of chemical substances or 'trophic' factors between developing cells have been implicated as playing central roles in (1) neural induction (for review, see Jacobson, 1978); (2) the differentiation of neurones derived from neural crest cells (Levi-Montalcini, 1976; Patterson, 1978; Black, 1978; Varon & Bunge, 1978; LeDouarin, Smith & LeLievre, 1981), and (3) the survival, development, and maintenance of central neurones and their target cells (Prestige, 1970; Cowan, 1970; Frank & Fischbach, 1979; Schacher, Kandel & Woolley, 1979*a*, *b*; Landmesser, 1981; Dennis, 1981; Harris, 1981). The external environment includes nutritive factors for proper growth and maintenance (Winick, 1976) and appropriate sensory stimuli during a critical period in early postnatal life (Hubel & Wiesel, 1970; Hubel, Wiesel & LeVay, 1975; Gottlieb, 1976) to stabilize connexions formed earlier in development. In this paper I will review briefly the role of neurone–glia interactions in neuronal development and describe how this interaction influences the *in vivo* development of abdominal ganglion neurones in *Aplysia californica*.

NEURONE–GLIA INTERACTIONS INFLUENCE NEURONAL DEVELOPMENT

Glial cells appear to influence neuronal differentiation in at least two ways. (1) Glial cells form a specifically orientated cellular substrate which can guide nerve cell bodies and axons to their appropriate regions. (2) Glial cells can release a variety of 'trophic' substances, including nerve growth factor (NGF), which influence the morphological and biochemical differentiation of neighbouring neurones.

In the developing central nervous system, the migration of various neuronal elements from proliferative zones to final positions proceeds along radially orientated fibres of glial cells (Ramon y Cajal, 1960; Rakic, 1971, 1972; Landis & Sidman, 1978; Nawakowski & Rakic, 1979) which ultimately differentiate into astrocytes (Schmechel

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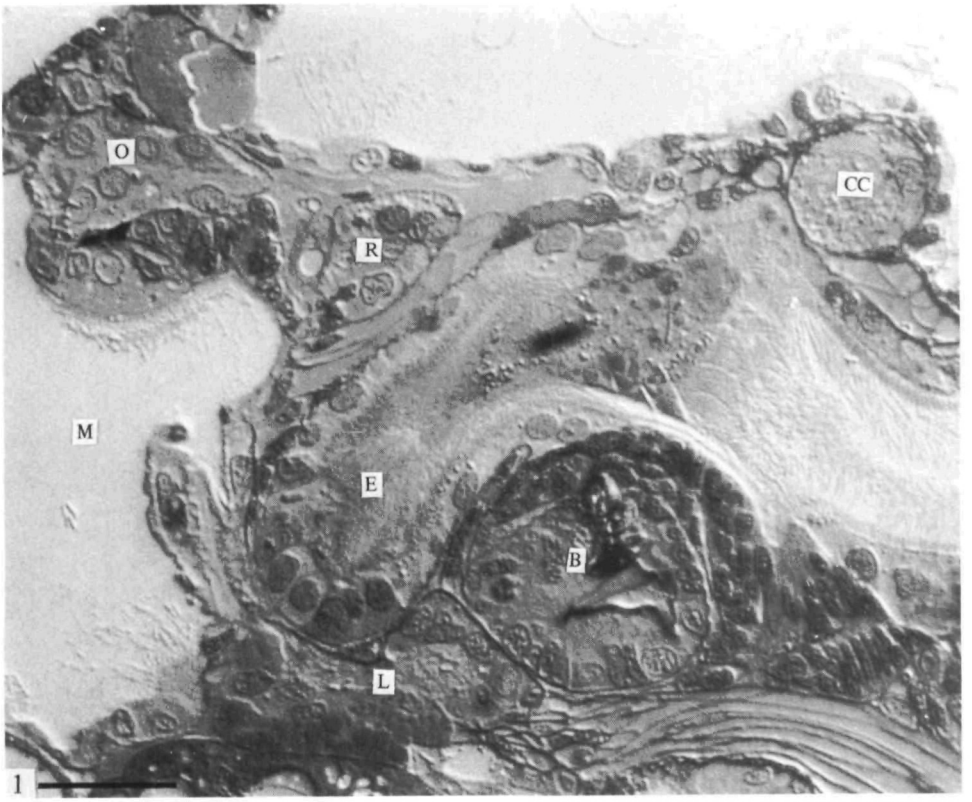
Fig. 1. Nomarski optics photomicrograph of a cross-section through a premetamorphic stage-6 animal at the level of the abdominal ganglion. The section was stained with toluidine blue. Large darkly staining granules can be seen at the perimeter of the left abdominal hemiganglion (L). Smaller and lightly staining granules can be seen within the right abdominal hemiganglion (R). O, Osphradial ganglion; E, oesophagus; M, mantle cavity; CC, cerebral commissure; B, buccal mass. The bar represents 10 μm.

Fig. 2. Portions of two non-neural support cells in the left abdominal hemiganglion. The cells have large membrane-delineated secretory granules which stain with varying intensities (G1 and G2), irregularly shaped nuclei (S), and extensive amounts of rough endoplasmic reticulum (RER). E, Extraganglionic space; n, nucleus of a ganglion neurone. The bar represents 1 μm.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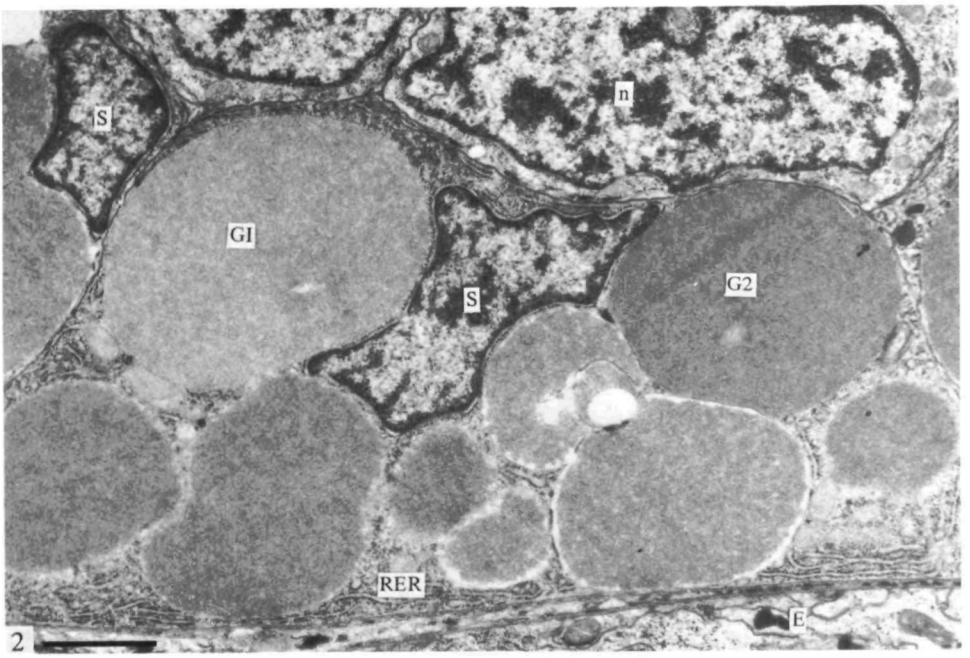
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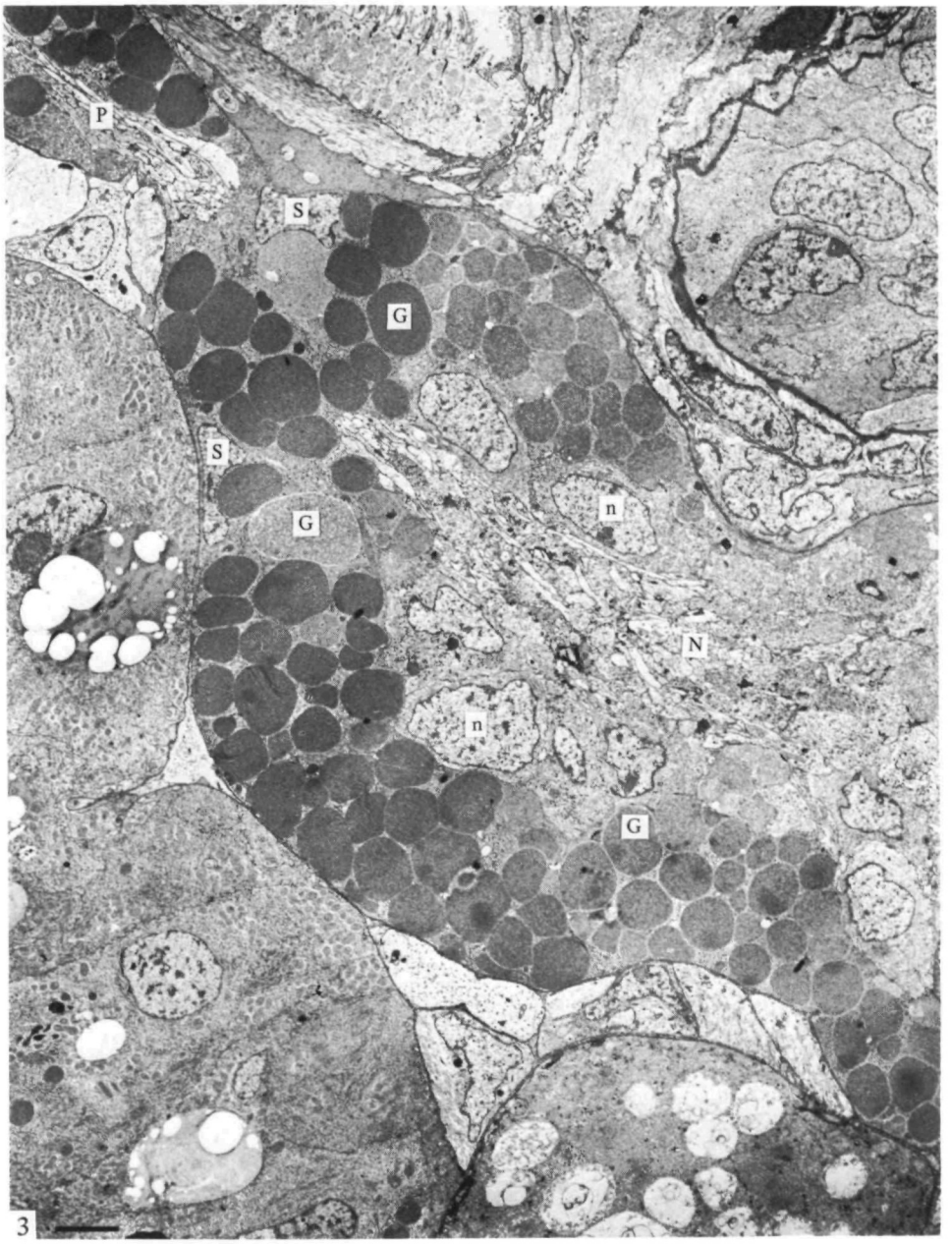

Fig. 3. Portion of the left abdominal hemiganglion in a premetamorphic stage-6 animal. The ganglion is embedded in a basket of granule-filled support cells. Some granules (G) are quite large (4 μm). Granule-filled processes also encapsulate the initial portion of the peripheral nerve (P). Support cell nuclei (S) can be seen near the peripheral nerve. N, Neuropile; n, ganglion cell nuclei. The bar represents 2 μm.

Rakic, 1979). The interaction between the migrating neurones and the glial cells may be critical for neurone differentiation. In two autosomal recessive mutations in mouse, 'weaver' and 'reeler', the disorientation and elimination of the Bergmann glial fibres could explain the degeneration of granule cells and the malposition of the cerebellar neurones (Rezai & Yoon, 1972; Rakic & Sidman, 1973*a*, *b*; Rakic, 1976; Caviness & Rakic, 1978).

Glial cells have been shown to release a variety of trophic substances which can influence neuronal differentiation (Patterson, 1978; Varon & Bunge, 1978; Mudge, this volume). Sympathetic neurones in culture can express atypical neurotransmitter synthetic pathways when grown in the presence of glial cells or medium conditioned by glial cells (Patterson, 1978). In addition, NGF is released by glial cells in culture which will permit dorsal root ganglion and sympathetic neurones to survive and grow processes even in the absence of exogenous NGF (Burnham, Raiborn & Varon, 1972; Varon, Raiborn & Burnham, 1974*a*, *b*; Ebendal & Jacobson, 1977).

NEURONE–SUPPORT CELL INTERACTIONS INFLUENCE THE DIFFERENTIATION OF ABDOMINAL GANGLION NEURONES IN APLYSIA

During the premetamorphic stages of development (stages 1–6; see Kriegstein, 1977*a*, *b*; Kandel, Kriegstein & Schacher, 1980, for description of the developmental stages of *Aplysia*) the ganglionic clusters are embedded within a capsule of support cells and their processes (Fig. 1). The major characteristic features of support cells are the presence of membrane delimited secretory granules which range in size from 0·5–5 μm in diameter, an irregularly shaped nucleus, and extensive amounts of rough endoplasmic reticulum (Fig. 2). The support cells are nonneural since they do not contain synaptic vesicles and membrane specializations that are characteristic of chemical synapses, and their processes contain few microtubules and filaments that are typically found in neurones.

Various histochemical stains suggest that the granules are basophilic and probably contain protein. The granules often stain intensely with a variety of basic dyes that stain protein. By contrast, the granules contain little lipid or polysaccharides since they do not stain with the periodic acid–Schiff stain and the Nile Blue or Sudan-Black stains.

When examined with the electron microscope, the granules stain with varying intensities (Figs. 2–3). These variations occur among the granules of a single support cell (Figs. 2–3). The variations may represent differences in the density of material within the granules. During the period in which granule material is released, the staining of the granules shifts from high to low intensity.

The support cells surrounding each ganglion are depleted of their granules at different times, with granule depletion from rostral ganglia preceding caudal ganglia. For example, at premetamorphic stage 5, the pedal, cerebral and pleural ganglia have only a few granules remaining in their support cells. By contrast, the abdominal ganglion contains support cells with numerous large granules at stage 6 (Fig. 3). The support cells of the abdominal ganglion have few granules following the metamorphic stage (Fig. 4).

In the earliest premetamorphic stages the granule-filled processes that surround the abdominal ganglion are not derived from support cells located in the ganglion. The support cells with cell bodies in the pleural ganglion have long processes containing granules which line the pleuroabdominal connectives and surround the abdominal ganglion. In late stage 3, additional granule-filled support cells whose cell bodies are in the abdominal ganglion appear. These cells are present in the peripheral regions of the ganglion and project granule-filled processes around some individual cells and the initial portion of peripheral nerves (Fig. 3).

The support cells surrounding the abdominal ganglion release their granule material after the animals settle on the appropriate macroalgal substrate which triggers the onset of the metamorphic phase (Fig. 4). The support cells become thinner and invaginate their processes between neurones to a greater extent. The support cells undergo two additional fine structural changes during metamorphosis-induced granule depletion. (1) Some of the discharged granule membrane is replaced initially by numerous small electron-lucent vesicles, multivesicular bodies and irregularly shaped membrane cisternae. By analogy to other secretory systems, these structures could represent the compensatory retrieval of excess membrane from the cell surface that occurs following the exocytic release of the granule contents. (2) The support cells begin to develop the morphological features characteristic of glial cells. Astrocyte-like fibre bundles or tonofilaments similar to those found in adult *Aplysia* glial cells can be seen forming in some cells which have not been depleted of all the granules.

The release of the granule material at metamorphosis coincides with an increase in cell body growth and synapse formation in the abdominal ganglion (Fig. 5). Metamorphic animals (Fig. 5 D) show a 85 % increase in cell body volume and a 100 % increase in morphologically identified active zones per unit area of neuropile compared to younger premetamorphic stage 6 animals (Fig. 5 A). Preventing metamorphosis and granule material release (the ganglion remains embedded in granule-filled support cells) by withholding the macroalgal substrate blocks half of the increase in cell body volume and synapse density associated with metamorphosis. Metamorphic animals show a 30 % increase in cell body volume and a 50 % increase in synapse density over premetamorphic stage-6 animals that are identical in age (Fig. 5 B).

Granule material release at metamorphosis can account for most of the increases in cell body growth and synapse formation associated with metamorphosis. Artificially inducing the release of the granule material while blocking metamorphosis by withholding the triggering substrate (Fig. 5 C) results in increases in cell body volume and synapse density over the other stage 6 groups (Fig. 5 A, 5 B) and are comparable to the increases seen in the metamorphic animals (Fig. 5 D).

Granule material release can be induced by exposing premetamorphic animals for 15–30 min to an artificial sea-water solution containing a high potassium concentration. Release in high potassium occurs even when the calcium concentration is reduced (Fig. 6). High-potassium/low-calcium treatment has the advantage that it apparently results in the selective release of material from support cells. Other secretory cells, such as gland cells in the foot or body wall, are unaffected. By contrast, in high-potassium sea water containing normal concentrations of calcium the other secretory cells in the animal also release their secretory contents.

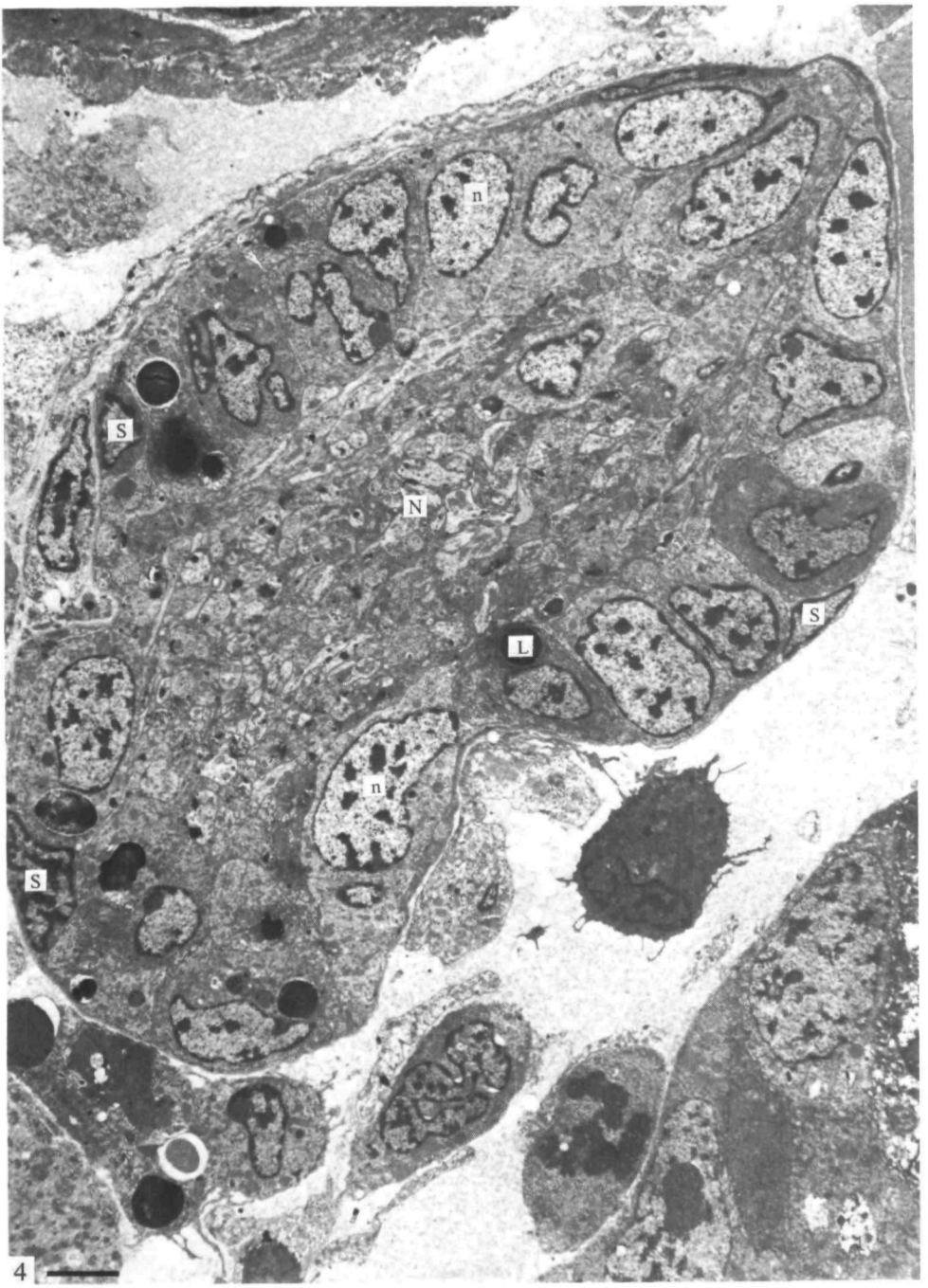

Fig. 4. Cross-section through the left abdominal hemiganglion in a stage-8 animal (48 h after the onset of metamorphosis triggered by the animal settling on the seaweed *Laurencia*). Virtually no granules are present within the support cells (S). Large densely staining bodies are lipid granules (L) within the ganglion cells (n). N, Neuropile. The bar represents 2 μm.

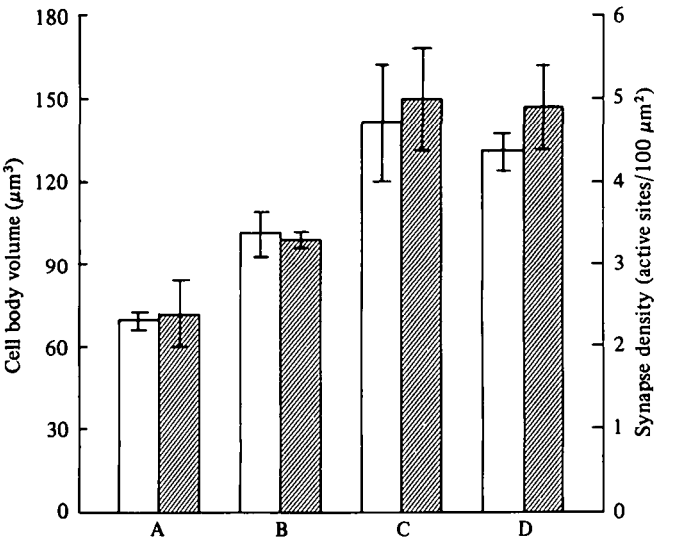

Fig. 5. Measurements of cell body volume (open bars) and synapse density (hatched bars) from three groups of premetamorphic stage-6 animals (A–C) and one group of postmetamorphic stage-8 animals (D). (A) Stage-6 animals before settlement on the triggering seaweed. (B) Stage-6 animals 1 week older than group A. (C) Stage-6 animals identical in age to group B but 1 week after pulse with high-potassium/low-calcium sea water. (D) Stage-8 animals identical in age to groups B and C but 1 week after settlement on seaweed that triggered metamorphosis. The height of each bar is the average of the means of measurements from four left abdominal hemiganglia (25 neurones per ganglia for cell body volume and 3000 μm^2 per ganglia for synapse density). A one-way analysis of the variance showed an overall significant difference between groups, $P < 0{\cdot}005$). Differences between individual groups were also significant for cell body volumes, and synapse density. Groups C and D are each significantly greater than Group A or Group B ($P < 0{\cdot}005$, Students' t-test, two-tailed). Group B measurements are significantly greater than group A measurements ($P < 0{\cdot}005$). Groups C and D are not significantly different. The error bars represent standard deviations.

The release of the granule material is not sufficient to produce all the changes in the abdominal ganglion that occur following metamorphosis. Metamorphosis, i.e. the settlement of the veligers on the appropriate substrate, is required for (1) support cell differentiation of glial properties (see above) and (2) the rapid growth of R2. This giant cholinergic cell in the abdominal ganglion shows a threefold increase in size immediately after metamorphosis which allows it to be identified for the first time on the basis of size and position (Kriegstein, 1977*a*). The selective growth and development of R2 is absent with granule release alone, although the general cell population in the ganglion shows a growth spurt comparable to metamorphic preparations (Fig. 5). Thus, the settlement of the animals on an appropriate external substrate can influence the development of the abdominal ganglion in at least two ways. (1) It signals the support cells to release the granule material producing a general growth spurt in the neuronal population. (2) It stimulates the growth and maturation of specific cells via a pathway that is independent of granule release from the support cells.

Another way to isolate the effects of granule release on neuronal development from metamorphic events is to artificially induce the release of the granule material in early

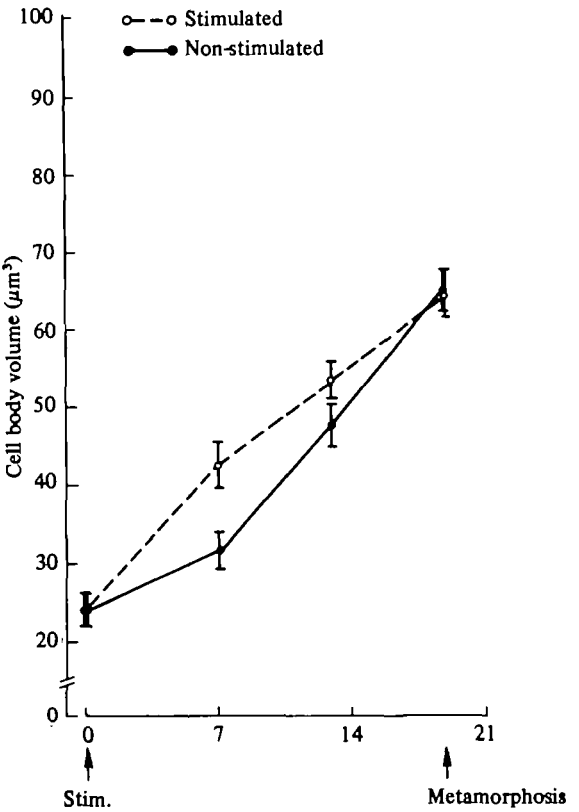

Fig. 7. The growth in cell body volume of right abdominal hemiganglion cells in control animals (closed circles) and in animals exposed for 15–30 min to high-potassium/low-calcium sea water (open circles). Each data point is the average of the means of measurements (20–25 cells per ganglion) from four ganglia. The experimental group is significantly greater than the control group ($P < 0.005$) at one week after pulse. The error bars represent standard deviations.

premetamorphic animals. Veligers exposed to high-potassium/low-calcium seawater results in the precocious maturation of the abdominal ganglion neurones. Animals in late stage 3/early stage 4 were exposed to the artificial seawater and allowed to continue development. The animals treated in this way showed increases in (*a*) cell body volume growth (Fig. 7), (*b*) synapse density within the neuropile (Fig. 8) and (*c*) spine and synapse formation by specific cells (Table 1).

One week after treatment, the average nerve-cell body volume in experimental animals is 30% greater than in control animals. This difference diminished in the following 2 weeks, so that at metamorphosis, cell body volumes are similar for both control and experimental animals (Fig. 7).

The premature release of granule material leads to a premature increase in synapse density within the neuropile (Fig. 8). Synapse density in control animals remains constant during the premetamorphic stages. By contrast, experimental animals show an increase in synapse density before metamorphosis at 2 and 3 weeks after premature release (65% and 90% respectively).

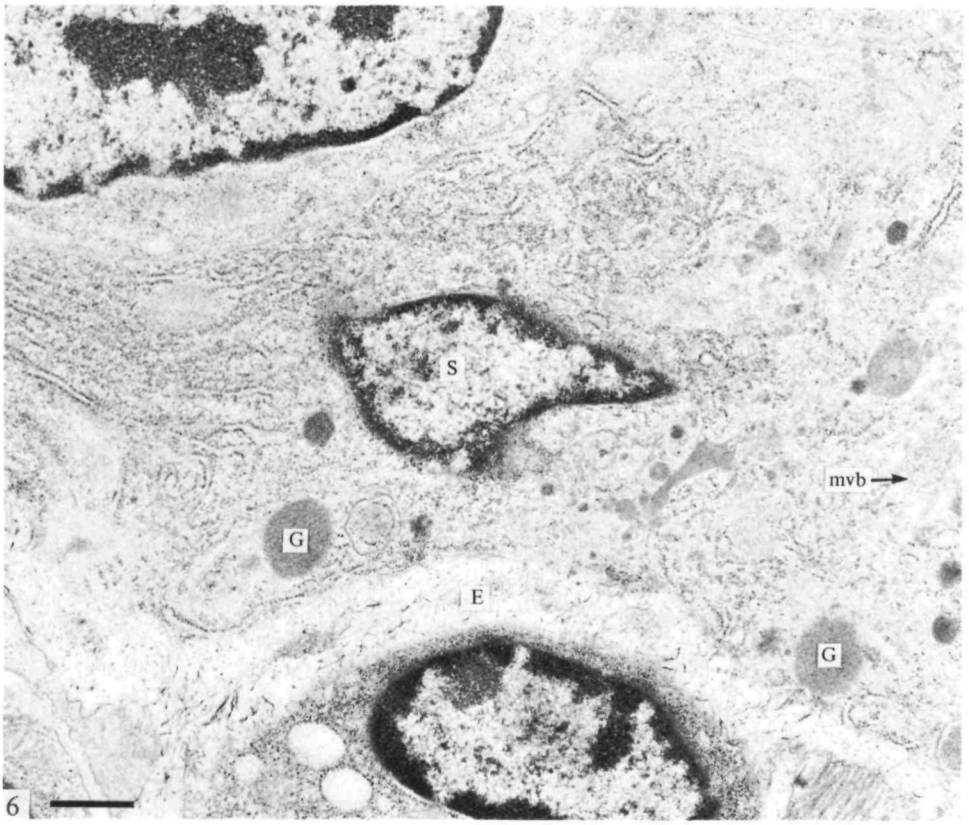

Fig. 6. Granule release from a nonneural support cell in a stage-3 animal that was exposed for 15–30 min to high-potassium/low-calcium sea water. The granules are absent with the exception of two unreleased granules (G) positioned close to the plasma membrane. S, Support cell nucleus; mvb, a multivesicular body; E, extraganglionic space. The bar represents 0·5 μm.

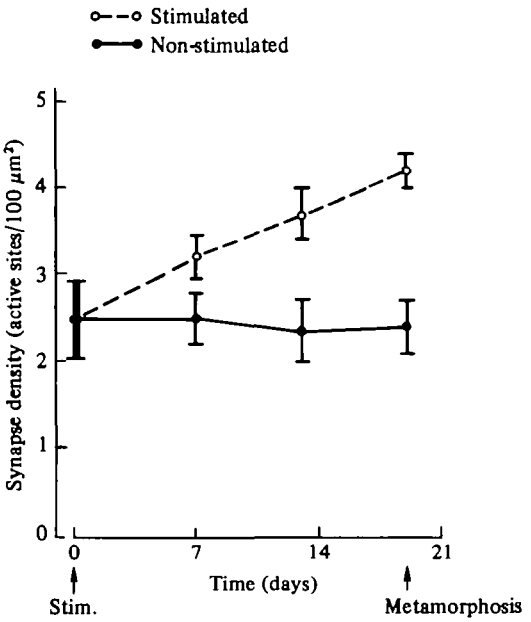

Fig. 8. The change in synapse density during development in the right abdominal hemiganglion in control animals (closed circles) and in animals exposed for 15–30 min to high-potassium/low-calcium artificial sea water (open circles). Each data point is the average of the means of synapse density measurements (active zones per 100 μm^2 of neuropile 1500–3000 μm^2 per ganglion) from four ganglia. The experimental group is significantly greater than the control group at 13 and 19 days after pulse ($P < 0.01$ and < 0.005 respectively).

Table 1. *Effect of premature granule release on identified cells*

	Spines		Synapses	
	Exp.	Control	Exp.	Control
Cell 1	11·3	5·7	4·7	0·3
Cell 2	12·0	4·7	3·3	0·3

The average number of spines, and synaptic contacts on the spines and major processes for two identified cells in the right abdominal hemiganglion of stage-6 animals. The experimental animals were treated with high-potassium/low-calcium artificial sea water 19 days earlier. N equals 3 for each cell in both experimental and control conditions. The esperimental cells have significantly greater numbers of spines and synapses ($P < 0.01$) than the control cells.

Precocious synapse formation due to premature release of granule material can also be analysed at the level of single cells. Two cells on the dorsal surface in the rostral portion of the right abdominal hemiganglion can be identified from animal to animal in the late premetamorphic stages (stages 5–6). In reconstructing their axons from serial thin sections, each cell in experimental animals has twice as many spines as does the same cell in control animals. Similarly, the number of morphologically identifiable synaptic contacts on the spines and the primary processes of the cells of the experimental animals is 10 times greater than in the same cells of control animals (Table 1).

These results suggest that the release of the granule material at metamorphosis has an important influence on the maturation of abdominal ganglion neurones. This neurone–glia interaction, and other types of interactions, such as neurone–neurone and neurone–target cell, may represent some of the timely epigenetic cues which permit both simple and complex neural networks to develop their unique properties.

CONCLUSION

The development of *Aplysia's* nervous system is an interesting example of how a combination of both external and internal environmental cues can influence neuronal differentiation. Unlike most other invertebrates and lower vertebrates, metamorphosis in *Aplysia* is triggered by the self-initiated behaviour of seeking out and settling on an appropriate seaweed (Kriegstein, Castellucci & Kandel, 1974). This precisely timed external factor then sets off a series of sequential cellular changes that enables the animal to develop adult phenotypes and express adult behaviours. Some of the adult behaviour that emerges soon after metamorphosis is controlled directly by identified abdominal ganglion neurones whose properties and interconnexions are known (Kandel, 1976). Thus, perturbations in the time of metamorphosis and the other cellular events which follow, i.e. the release of the granule material from the support cells, could strongly influence the way these specific neurones interconnect with each other and differentiate their unique properties. This type of analysis could lead to an understanding of the developmental programme underlying the emergence of the neural control of an animal's behavioural repertoire.

I wish to thank Thomas Capo and Susan Perritt for their work in raising the animals at the Marine Biological laboratory at Woods Hole. I also thank Robert Woolley and Kathrin Hilten for their technical assistance. This work was supported by a grant from the National Science Foundation BNS 78-23602 and a Public Health Service Grant NS 15279.

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