

## Stimulation of Shell Regeneration by Crude Extract of Subesophageal Ganglionic Mass in Giant African Snails, *Achatina fulica* (Bowdich)

Viyada Seehabutr

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### ABSTRACT

The distribution of cells that stained positively with paraldehyde fuchsin (PAF) and negatively with chrome-hematoxylin phloxine (CH) in the subesophageal ganglionic mass of *Achatina fulica* has been mapped. The PAF-positive cells occur in the visceral ganglion, each cell contains electron-dense elementary granules of 1,300 Angstrom in diameter. From serial section, it showed that the PAF-positive cells or neurosecretory cells (NSC) sent axons into the intestinal nerve. The CH-negative cells occurred in the right parietal ganglion, each cell contained electron-dense elementary granules of 1,370 Angstrom in diameter. From serial sections, it showed that CH-negative cells sent axons into the pallial nerve.

PAF-positive and CH-negative materials were depleted from cells in 24 hr after shell removal and reappeared by 72 h. Shell regeneration apparently completed within 15 days. Moreover, the subesophageal ganglionic mass homogenate (SGH) had some effects on shell regeneration which suggested that the PAF-positive and CH-negative cells were the neurosecretory cells that control shell regeneration.

**Key words:** neurosecretory cells, paraldehyde-fuchsin, chrome-hematoxylin phloxine, pallial nerve, shell regeneration, subesophageal ganglionic mass homogenate

### INTRODUCTION

*Achatina fulica* is a land snail. It belongs to the phylum Mollusca, class Gastropoda, subclass Pulmonata, order Stylommatophora, family Achatinidae. These land snails are plentiful in the tropical countries with high rainfall. The shell of *A. fulica* consists of 7 to 12 whorls, with moderately swollen body whorl and a sharply conical spire, which is distinctly narrowed but scarcely drawn out at the apex. The snail has no gill and operculum, but the mantle cavity serves as a lung. It has two pairs of retractile tentacles, with eyes at the tips of posterior tentacles.

The nervous system of *A. fulica* consists of

13 ganglia; a pair of buccal ganglia, a pair of cerebral ganglia, a pair of tentacular ganglia and a subesophageal ganglionic mass. The subesophageal ganglionic mass is a mass of nervous tissues lies under the esophagus and columellar muscle. It is composed of seven ganglia; two pleural ganglia, two parietal ganglia, two pedal ganglia and a single visceral ganglion.

Seehabutr (1992) reported that there are neurosecretory cells in procerebrum of cerebral ganglia of *A. fulica*. The crude extract of these ganglia has effects on oogenesis in the ovotestis of *A. fulica*. In this experiment, the possible role of neurosecretory cells on shell regeneration in *A. fulica* has been examined.

## MATERIAL AND METHODS

### 1. Histochemical preparation for morphological study of the neurosecretory cells by light micro-scopy

#### 1.1 Experimental animals and paraffin embedment

Mature *A. fulica* with the shell height of 7 cm were used in this experiment. They were collected from the field during the rainy season. Subesophageal ganglionic mass were removed from snails and processed for serial paraffin sections. The 6 µm thick serial sections were then submitted for further staining. Chrome-hematoxylin phloxine and paraldehyde-fuchsin were used to identify the neurosecretory cells in subesophageal ganglionic mass.

#### 1.2 Chrome-hematoxylin phloxine staining method (Gomori, 1941).

Subesophageal ganglionic mass were fixed in Bouin's fluid and processed through paraffin section at 6 µm thick. The section were tested for about 1 min with a solution containing about 0.3% each of potassium permanganate and sulfuric acid, then decolorized with a 2 to 5% solution of sodium bisulfite. After staining with hematoxylin solution, the sections were counterstained with 0.5% aqueous solution of phloxine (B) (Gomori, 1941).

#### 1.3 Paraldehyde-fuchsin staining method (Gomori, 1950).

The paraffin sections (6 µm thick) were deparaffinized, hydrated and oxidized in Gomori's fluid. After staining in paraldehyde-fuchsin solution the sections were counterstained in Halmi's mixture (Cameron and Steele, 1959). Then, the sections were examined under the bright field of microscope.

### 2. Histological preparation for ultrastructural study of the neurosecretory cells by transmission electron microscopy

The specimens of subesophageal ganglionic mass were embedded in araldite. The plastic blocks of tissue were sectioned using glass knives and

stained with uranyl acetate, and counterstained with lead citrate. Then the samples were examined using Hitachi H-300 electron microscope.

### 3. Subesophageal ganglionic mass homogenate preparation

Adult *A. fulica* (about 5 whorls, 5-6 cm long and 15-20 g in weight) were collected from the wild during May to October, 1999. These snails were anesthetized with nembutal for 30 minutes. The subesophageal ganglionic mass were dissected, cut into small pieces and frozen on dry ice before extraction. Subsequently, this ganglionic tissue was ground in normal saline; pH 8.5 using hand homogenizer (Pelluet and Lane, 1961) (the one milliliter of saline was used to extract 2-3 ganglionic mass). The sample was then centrifuged at about 3,000 g for 20 minutes. The clear supernatant was collected.

### 4. Experimental design

Adult snails (14-18 g in weight) were collected from the wild and kept in the containers for 7 days before testing. The snails were divided into 3 groups (30 snails /group); the control group, the sham-operated group (received 10 µm of normal saline per snail once a week for 4 weeks by oral method), the experimental group [received subesophageal ganglionic mass homogenate (2 mass/snail) once a week for 4 weeks by oral method]. To initiate shell regeneration, a piece of 1 cm<sup>2</sup> was removed at the growing edge.

All the snails were kept in the plastic container at 22°C under 12 Light : 12 Dark photoperiod. The diet was supplied *ad libitum*. The weight and shell regeneration of each snail were recorded every week throughout the experiment.

## RESULTS

### Distribution of neurosecretory cells

The distribution of neurosecretory cells in the subesophageal ganglionic mass of *A. fulica* that

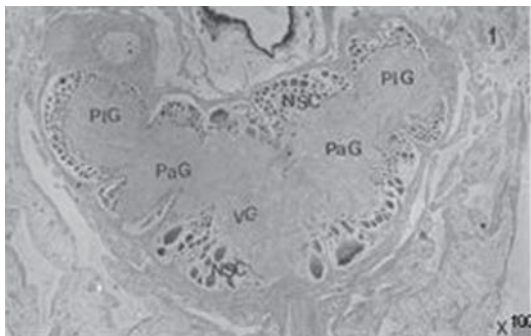
stained with PAF and CH were shown in Figures 6-7.

PAF-positive cells were found in the visceral ganglion. There were about 15 cells with an average individual diameter of 50-70  $\mu\text{m}$  lying posterior to the visceral-parietal connective tissue (Figure 1). At the ultrastructural level these cells were found to contain electron dense granules of 1,300 Angstrom in diameter (Figure 2). The cell bodies contain prominent rough endoplasmic reticulum and accumulations of glycogen and numerous golgi complexes. Large dense bodies resembling lysosomes varied in abundance.

CH-negative cells were found in the right parietal ganglion. There were about 30-36 cells with an average individual diameter of 60-80  $\mu\text{m}$  lying in the anterior part of the ganglion (Figure 1). At the ultrastructural level these cells were found to contain electron dense granules of 1,370 Angstrom in diameter (Figure 3). The organelles in the cells bodies were the same as PAF-positive cells of visceral ganglion.

### Changes in the neurosecretory cells during shell regeneration

After removing a large segment of the shell from the growing edge, regeneration appeared to be completed within 15 days. Twenty-four hours after

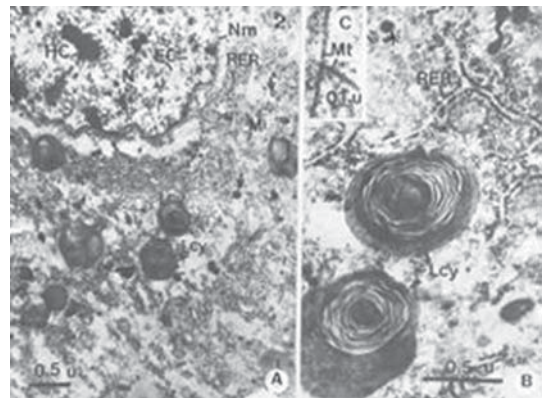


**Figure 1** subesophageal ganglionic mass

PIG = pleural ganglion

PaG = parietal ganglion

VG = visceral ganglion



**Figure 2** A-B-C TEM Micrograph of visceral ganglion

N = nucleus

HC = heterochromatin

EC = euchromatin

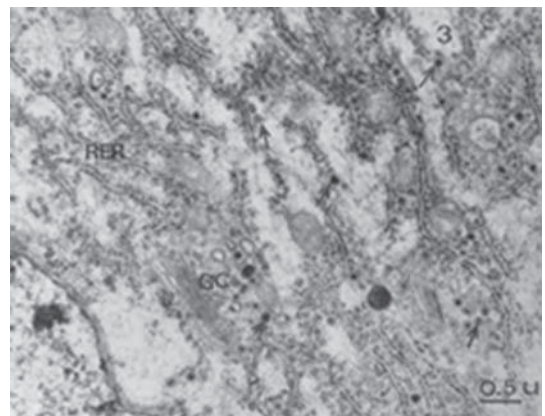
Mi = mitochondria

Lcy = lysosome

RER = rough endoplasmic reticulum

Mt = microtubule

arrow = electron dense elementary granule



**Figure 3** TEM Micrograph of parietal ganglion

RER = rough endoplasmic reticulum

GC = golgi complex

arrow = electron dense elementary granule

shell removal the cell bodies in visceral and parietal ganglia showed reduced staining reduction, or none at all, with PAF and CH, whereas the right pallial and the intestinal nerves showed dark staining with PAF and CH (Figure 5-6). However the stained material begins to reappear in the cell bodies of visceral and parietal ganglia about 72 hours after shell removal (Figure 6, 7).

### Effect of subesophageal ganglionic mass homogenate (SGH) on shell regeneration

One week after treating with normal saline and SGH, the width of regenerated shell of control group, normal saline group and SGH group were 2.3 mm, 2.94 mm and 3.5 mm, respectively.

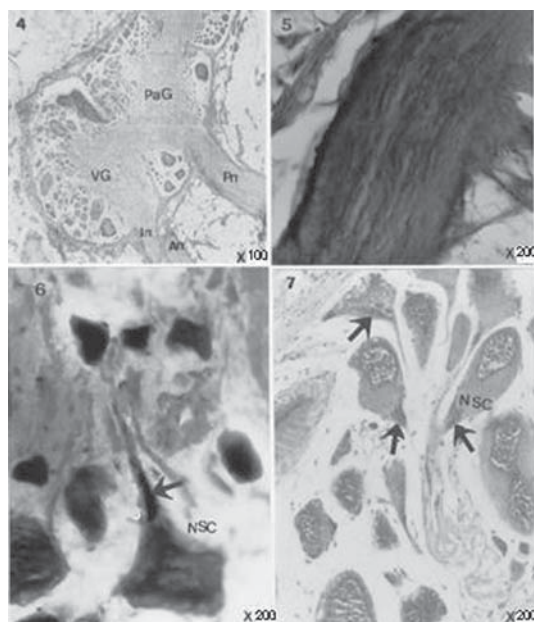
In the second week, the average width of regenerated shell of the control group, normal saline group and SGH group were 8.2 mm, 7.1 and 9.2 mm, respectively.

The shell regeneration of all groups was complete in the third week after treatment. However the shell regeneration of SGH treated group was prior and thicker (0.35 mm) than those of the control group (0.28 mm) and normal saline groups (0.27 mm).

## DISCUSSION

A correlation has been demonstrated between changes in the histological appearance of neurosecretory cells in subesophageal ganglionic mass (VG, PaG) and the early stages of the shell repair after damage in *A. fulica*. This correlation suggests a possible role of neurosecretory material in the control of shell repair. This finding agrees with the work of Dillaman *et al.* (1976) on the neurosecretory cells in visceral ganglion having some effects on shell regeneration.

Saleuddin (1975) reported that the mantle edge gland, which is responsible for laying down the shell material, is supplied by neurosecretory axons and it is possible that the axons that run in the right pallial nerve may innervate the mantle edge



**Figure 4** subesophageal ganglionic mass

Pag = parietal ganglion

VG = visceral ganglion

In = intestinal nerve

An = anal nerve

Pn = pallial nerve

**Figure 5** Intestinal nerve

arrow - axons of neurosecretory cells

**Figure 6** Neurosecretory cells (PAF-positive material)

**Figure 7** Neurosecretory cells (CH-negative cell) of parietal ganglion

arrow = neurosecretory material (CH-negative material)

directly (Kerkut and Walker, 1975). So, it could be concluded that the neurosecretory cells in PaG might control the shell regeneration in *A. fulica*. Moreover, there are some neurosecretory cells in visceral ganglion and their axons accumulated neurosecretory material running into the intestinal nerve. This finding agrees with the research of Simpson (1969) that most of the neurosecretory axons from the visceral ganglion run in the intestinal nerve, terminating partly in the aorta and partly in

the heart.

Information on the effect of SGH on shell regeneration of *A. fulica* could lead us to determine the location and action of NSC in PaG and VG.

### CONCLUSION

The cells that stained positively with paraldehyde fuchsin (PAF) could be found in the visceral ganglion and the cells that stained negatively with chrome-hematoxylin phloxine (CH) could be found in the right parietal ganglion. The subesophageal ganglionic mass homogenate has some effects on shell regeneration of *A. fulica* after administration by oral method.

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