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IDENTIFICATION OF CENTRAL NEURONS INNERVATING THE HEART OF *LYMNAEA STAGNALIS* L. (GASTROPODA)

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The question whether only the abdominal ganglion or other ganglia are also involved in the regulation of heart activity of Gastropods has long been discussed. According to VAN TIEL (1942) the nervous centres inhibiting and stimulating heart activity are located in the abdominal ganglion, while KOSHTOYANTZ, SMIRNOVA and POPKOVA (1956) believe the abdominal ganglion to be the centre of inhibition and the cerebral ganglion the site of initiation of both inhibitory and excitatory effects.

For the identification of central neurons from which direct fibres run to the heart, the method of COHEN and JACKLET (1965), which proved to be useful with Molluscs (SALÁNKI and GUBICZA (1967) seemed to be suitable. The method is based on the observation that axon regeneration is associated with accumulation of RNA in the central neurons which can be visualized with malachite green-pyronine staining.

In the present work those central neurons of *Lymnaea stagnalis* are described in which RNA accumulation were noted after the cutting through of the intestinal nerve.

Material and method

Experiments were performed on medium-sized specimens of *Lymnaea stagnalis* L. anaesthesized in 0,05—0,08 % solution of nembuthal until complete relaxation was achieved. Then the central nervous system was cut through the branch of the intestinal nerve innervating the heart was sectioned. After this surgical intervention the animals were placed in aquaria containing oxygenized circulating Balaton water for a period of 1—3 days. Only the specimens that survived operation were used for histological preparations.

The central nervous system was fixed in CARNOY's solution for 45 minutes. After the orientation of the ganglia, the material was embedded in paraffin and serially sectioned (thickness of sections : 7μ). Sectioning was always made in a horizontal plane in relation to the animal's horizontal posture. The sections were stained with mixture of malachite green (Edward Gurr, No 315) and pyronine y (PMaG) (GT Gurr, England) according to the method of BAKER and WILLIAMS (1965) modified by SALÁNKI and GUBICZA (1967).

The sections were examined under the light microscope. Comparative measurements were made on the size of neurons showing signs of regeneration and on their localization.

According to their size the nerve cells were classified in three groups:

large (120 μ or larger)

medium-sized (from 50 to 120 μ)

small (under 50 μ)

To facilitate localization of the cells the sections prepared from the ganglia were divided into three zones:

Zone I = ventral third of the ganglion

Zone II = median third of the ganglion

Zone III = dorsal third of the ganglion

By this division localization in the three zones of the neurons showing signs of regeneration was made possible *(Fig. 9)* on the basis of sections belonging to the given zone.

Parallel with the histological preparation of the material obtained from the operated animals, similar preparations were made from the nervous system of intact animals. Classification of cells according to size was performed in the control group, too. This method proved to be useful in evaluating the size and localization of nerve cells showing signs of regeneration. The percentage ratio of pyroninophilic cells was expressed by the method described by SALÁNKI and GUBICZA (1967) on *Anodonta cygnea.*

In order to see whether an increase of RNA occurs owing to operative trauma, additional control examinations were performed on the central nervous system of animals in which a skin section similar to that of the operated animals was made. The experiments were conducted in May and June on specimens of *Lymnaea stagnalis* collected from the Balaton 1–2 days prior to operation.

In the present work the results obtained on 8 animals in which nerve section was made, 8 controls and 4 animals with skin section are reported.

Results

1) *PMaG staining of central neurons in control animals*

Staining with PMaG yielded a faint homogeneous reaction in the cytoplasm of central neurons of the control animals. The so-called perinuclear ring localized around the nuclei of certain neurons in the central nervous system of *Lymnaea stagnalis* stained intensively with pyronine. The neurons possessing a perinuclear ring have small nucleoli and no RNA granulesare present in their nuclei *(Fig. 1)*. The number of such cells was found to vary in the experimental animals and their presence was not always demonstrable. They are usually scattered in various areas of the nervous system displaying no regular localization pattern.

The cytoplasm of some cells in the central nervous system of *Lymnaea stagnalis* contains RNA granules of 1–5 μ diameter *(Fig. 2)*. Neurons containing such RNA particles are usually arranged in groups and are most frequently found in the cerebral ganglion. In other ganglia they are seldom encountered. No regularity was observed in the localization of nerve cells rich

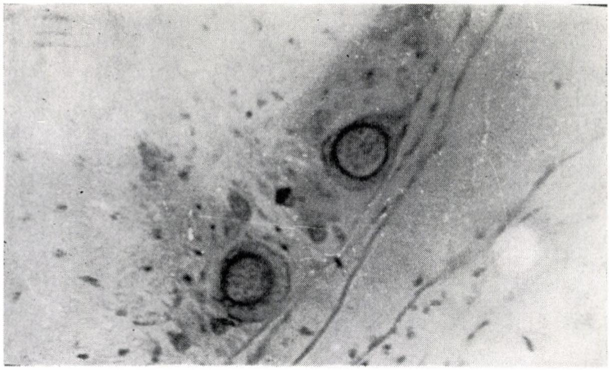

Fig. 1. Small pyroninophilic granular nerve cell in the cerebral ganglion of control *Lymnaea stagnalis*. ×320

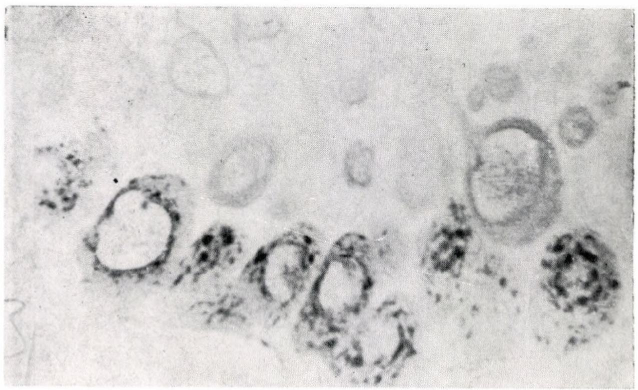

Fig. 2. RNA granules in the cytoplasm of neurons in the cerebral ganglion of control *Lymnaea stagnalis* ×320

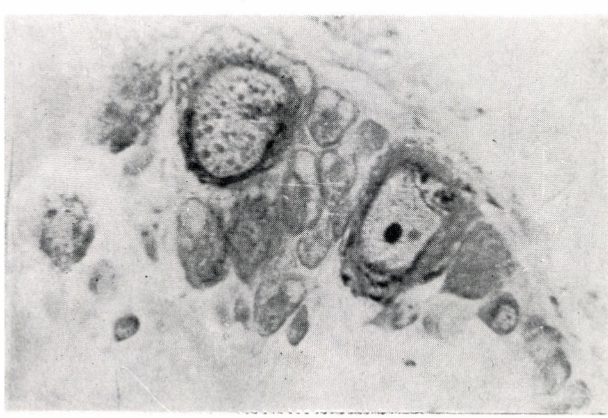

Fig. 3. Characteristic large nucleolus in the nerve cell of the abdominal ganglion of control *Lymnaea stagnalis* ×320

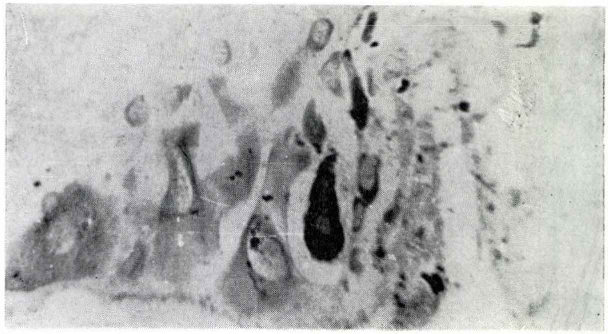

Fig. 4. Small (25×50 μ) pyroninophilic nerve cell in the right cerebral ganglion $\times 400$

Fig. 5. Small pyroninophilic granular neuron among the cells of the frontal lobe of the cerebral ganglion. $\times 320$

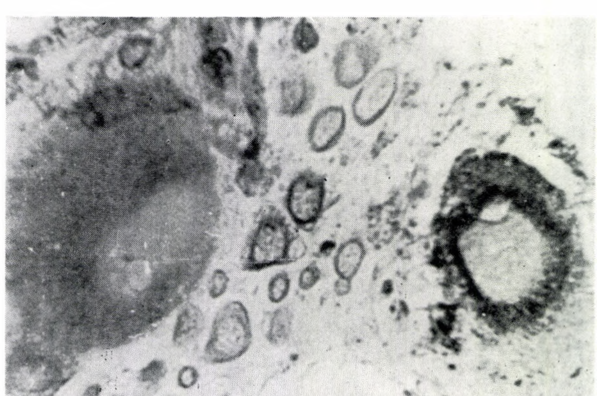

Fig. 6. Small (40 μ) pyroninophilic granular neuron in the right parietal ganglion. $\times 450$

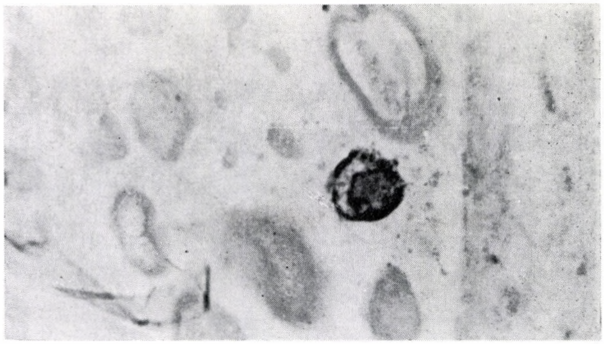

Fig. 7. Small (40 μ) pyroninophilic neuron in the right pedal ganglion. $\times 450$

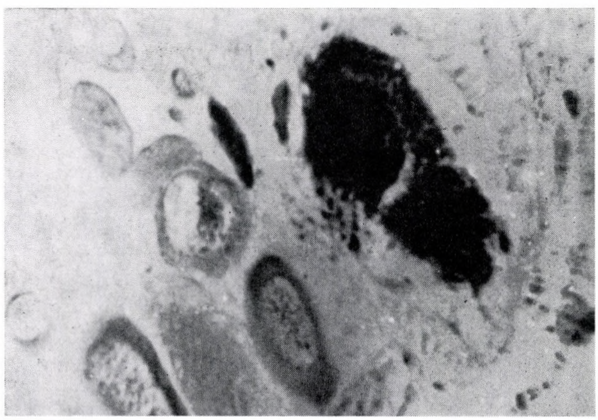

Fig. 8. Large pyroninophilic granular nerve cell in the abdominal ganglion $\times 320$

in RNA, their presence is sometimes undemonstrable. In the control animals the neurons with no perinuclear pyroninophilic ring were found to contain large nucleoli (3—11 μ), as shown in *Fig. 3.*

The amount of RNA granules in the nervous system of animals in which a similar skin section was performed as in animals submitted to nerve injury, corresponded to the amount of RNA found in the intact controls. In their neurons the perinuclear ring, RNA granules appearing irregularly in the cytoplasm and large nucleoli were demonstrable. No changes in the cytoplasmic RNA could be observed in the central neurons in this group of animals.

2) *Pyroninophilia and localization of axon-damaged neurons*

After axon injury the cytoplasm of some nerve cells was found to contain a granular substance staining well with pyronine y *(Fig. 4)*. The nuclei of these cells exhibiting granular pyroninophilia stained more intensively with malachite green than the nuclei of control cells. The nucleoli were inferior in size compared with the large nucleoli characteristic of the intact cells. No pyroninophilic cells possessing the characteristics described above were encountered in any of the neurons of the control animals. Aggregation of pyroninophilic material observed soon after nerve section was absent in the cytoplasm of the neurons if the sections were incubated with ribonuclease. Removal by the enzyme of the pyroninophilic material confirms its RNA nature.

On the basis of these observations granular pyroninophilia of the cytoplasm was accepted as sign of regeneration in the central nervous system of *Lymnaea*. As early as after 24 hours following nerve section the typical pattern of granular pyroninophilia could be observed and 56 hours after operation these cells were in a state of disintegration *(Fig. 5)*.

Localization of neurons exhibiting granular pyroninophilia after the intersection of the intestinal nerve is summarized in *Fig. 9*. The typical granular pyroninophilia was found to appear always in identic cells of the animals submitted to nerve injury. Our data have verified that the right and left pleural ganglia do not contain neurons exhibiting granular pyroninophilia. The cells containing pyroninophilic granules have an assymetrical localization in the central nervous system. In the left ganglia — except the pedal ganglion — there were fewer granular cells and pyroninophilic granulation was visible only in the giant cells of the right ganglia *(Fig.* 6, 7, 8).

The lowest number of pyroninophilic neurons was observed in the ventral part (zone I) of the ganglia. In this area positive reaction with pyronine y was obtained only in the right and left cerebral, and pedal ganglia. In the middle part of the ganglion (zone II) the highest number of neurons showing signs of regeneration was observed. In the basal part (zone III) pyroninophilic neurons were absent in the pleural and pedal ganglia. Concentration of RNA granules were demonstrated only in both cerebral and parietal ganglia, as well as in the abdominal ganglion *(Fig. 9)*.

In addition to the distribution in depth, localization of neurons showing signs of regeneration can be facilitated by the observation that in the abdominal ganglion, at the site of origin of the intestinal nerve, 4—5 pyroninophilic cells are always visible, while another characteristic group of cells is found on

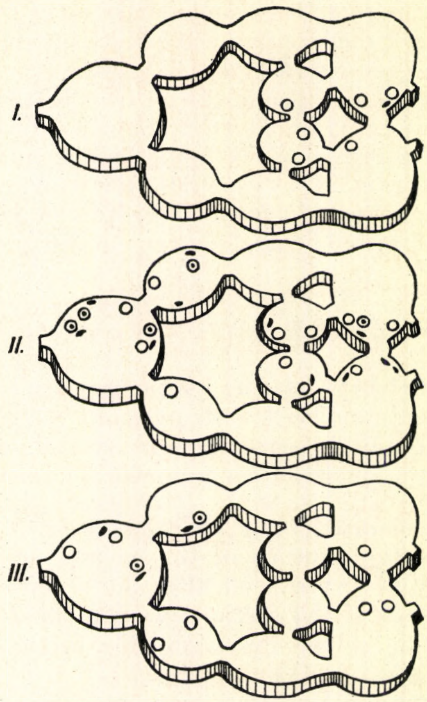

Fig. 9. Scheme of localization in the ganglia of neurons showing signs of retrograde regeneration in sections prepared from material varying in depth.
I = ventral zone
II = median zone
III = dorsal zone
⊙ = large neurons (over 120 μ)
O = medium-sized neurons (50—120 μ)
— = small neurons (under 50 μ)

the opposite side of the ganglion, at the issue of the parieto-abdominal connective. In the basal part (zone III) of the left parietal ganglion a giant cell showing granular pyroninophilia was found in each animal examined *(Fig. 6)*. In the cerebral ganglia most of the pyroninophilic cells were found around the site of origin of the commissura cerebro-cerebralis and in the area of procerebrum. *Table 1* shows the numerical distribution of neurons sending axons to the intestinal nerve.

According to the results of our examinations the number of neurons sending direct fibres to the heart of *Lymnaea* is 46. The percentage ratio of medium-sized, small and large neurons is 52,2, 32,6 and 15,2, respectively. In the right and left cerebral ganglion the total number of neurons involved (15) is superior to the number of reacting cells in the abdominal ganglion (13), though the nerve branch running to the heart takes origin from the latter ganglion. In the parietal and pedal ganglia identic number of neurons (9) are involved.

The fibres of the intestinal nerves consist mostly of small and medium-sized cells. Considering, however, that in the nervous system of *Lymnaea*

Table 1

Numerical distribution of nerve cells showing signs of regeneration, in the central nervous system of *Lymnaea*

Ganglion		Number of neurons			Total:
		large	medium-sized	small	
Cerebral	left	1	5	2	15
	right	—	4	3	
Pleural	left	—	—	—	
	right	—	—	—	
Parietal	left	2	1	3	9
	right	—	3	—	
Pedal	left	—	3	1	9
	right	—	4	1	
Abdominal		4	4	5	13

1,5—10% of the total neurons is composed of giant cells, 22—37% of medium-sized and 72% of small cells, a more important role must be attributed to giant cells than that shown by this percentage ratio in the regulation of heart activity because nearly one third of the giant cells send direct fibres to the intestinal nerve.

The percentage of giant cells involved in the regulation of heart activity is the highest in the abdominal ganglion directly innervating the heart. Compared ot their total number, the participation of small cells in the composition of the intestinal nerve is of the slightest degree.

Discussion

Central neurons of *Lymnaea stagnalis* following axon injury can be identified by staining with malachite green pyronine, similarly to *Anodonta* (SALÁNKI and GUBICZA, 1967). In the *Lymnaea* the appearance of the perinuclear ring of RNA is, however, not the sign of axon regeneration but it is believed to be a symptom concomitant with normal physiological processes of the animal. This assumption is supported by the data of BOER (1965) who described different types of RNA aggregates in the nervous system of *Lymnaea*, correlating them with the secretory activity of the nerve cells. According to BOER the perinuclear ring consisting of fine granules of RNA is characteristic of GOMORI-positive cells (BOER, 1965). In a terrestrial snail *(Zachryssia guanensis)* the RNA ring was found to disappear during hibernation. This finding seems to lay stress on the correlation of RNA concentration and activity of the animal (URBÁ-HOLMGREEN and HOLMGREEN, 1968).

The sign of regeneration in the neurons of *Lymnaea* is the considerable increase of RNA granules in the cytoplasm. This kind of RNA concentration differs distinctly from that noted in normal ganglia *(Fig.s 1,2* and *4)*. Digestion of this material by ribonuclease undoubtedly indicates the presence of a specific RNA accumulation.

On the basis of our experimental data it may be concluded that the two cerebral, the two parietal, the abdominal and the two pedal ganglia send direct fibres to the intestinal nerve branch innervating the heart. Considering the number of cells involved the cerebral ganglia seem to have the largest share (15 cells), then the abdominal ganglion (13 cells) and finally the parietal and pedal ganglia (9 cells each).

Our results show that the theory of the exclusive role of the abdominal ganglion in the regulation of heart activity in Gastropods (Van Tiel, 1942) is untenable. We have demonstrated that in addition to the abdominal and cerebral ganglia, the parietal and pedal ganglia are also involved in this regulation *(Fig. 9)*. Our examinations confirm the statement of Willows (1968) on the participation of giant cells in the control of peripheral organs. We have also found that the percentage of giant cells involved in the regulation of heart activity was the highest compared to their total number.

According to our data there is a morphological basis for the formation inhibitory and excitatory centres also in the pedal and parietal ganglia. The inhibitory of excitatory nature of neurons identified morphologically can, however, be clarified only after further pysiological investigations.

Summary

1. Axon-injured neurons in the central nervous system of *Lymnaea stagnalis* can be identified on the basis of granular RNA accumulation demonstrable in their cytoplasm. This granular RNA can be fairly distinguished from the homogeneous fine RNA granules present in normal animals.

2. Following intersection of the intestinal nerve specific RNA accumulation were found in the neurons of cerebral, parietal, abdominal and pedal ganglia. In all, 46 neurons emit direct fibres to the intestinal nerve.

3. Appearance of cytoplasmic concentration of RNA following intersection of the intestinal nerve is an evidence that in the regulation of heart activity the giant cells have the largest share in proportion to their total number.

4. In addition to the cerebral and abdominal ganglia, parietal and pedal ganglia are also involved in the regulation of heart activity in Gastropods.

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GASTROPODÁK SZÍVÉT BEIDEGZŐ KÖZPONTI NEURONOK AZONOSÍTÁSA

Gubicza András és *S.-Rózsa Katalin*

Összefoglalás

1. *Lymnaea stagnalis* központi idegrendszerében az axonkárosított neuronok identifikálhatók a citoplazmájukban kimutatható szemcsés RNS felhalmozódás alapján. E szemcsés RNS felhalmozódás jól elkülöníthető a normál állatokban is megtalálható homogén, apróbb szemcsés RNS képződményektől.

2. A szívet innerváló intestinális ideg átmetszése után a cerebrális, parietális, abdominális és pedális ganglionokban találhatók specifikus RNS felhalmozódást mutató idegsejtek. Összesen 46 neuron küld direkt rostot az intestinális idegbe.

3. Az intestinális ideg átmetszése után megfigyelt RNS felhalmozódás azt bizonyítja, hogy a szívműködés regulálásában össz-számukhoz viszonyítva az óriássejtek vannak legnagyobb arányban képviselve.

4. A cerebrális és abdominális ganglionokon kívül a parietális és pedális ganglionok is részt vesznek a szívműködés szabályozásában Gastropodákon.

ЛОКАЛИЗАЦИЯ ЦЕНТРАЛЬНЫХ НЕЙРОНОВ ИННЕРВИРУЮЩИХ СЕРДЦЕ БОЛЬШОГО ПРУДОВИКА (БЮХОНОГИЙ)

А. Губица и *К. Ш.-Рожа*

1. В центральной нервной системе большого прудовика можно идентифицировать нейроны после перерезки их аксонов на основе накопления зерен РНК в их цитоплазме. Это зернистое накоплене РНК резко отличается от мелкозернистого, гомогенного скопления РНК, характерного для контрольных животных.

2. После перерезки интестинального нерва иннервирующего сердце клетки указывающие специфическое скопление РНК обнаруживаются в церебральных, париетальных, педальных и абдоминальном ганглиях. Всего 46 клеток посылают аксоны в интестинальный нерв.

3. Полученные результаты указывают на то, что так называемые гигантские клетки участвуют в наивысшем проценте в регуляции сердца по сравнению их общим числом.

4. Кроме абдоминального и церебральных ганглиев париетальные и педальные ганглии тоже участвуют в регуляции сердечной деятельности Брюхоногих.