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RNA IN THE GANGLIA OF MOLLUSCA IN NORMAL CONDITIONS AND FOLLOWING NERVE DAMAGE (A HISTOCHEMICAL STUDY)

JÁNOS SALÁNKI and ANDRÁS GUBICZA

Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

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The nucleic acid content of nerve cells, its changes, and the closely related protein synthesis serving as a basis in cell function and regeneration have been subjected to detailed investigations (HYDEN 1947, 1960, 1962, BRODSKY 1966). Histological and histochemical examinations presented data first of all on the role of the nucleus and nucleolus, on the ratio of the dimensions among various parts of the cell, further on, the character and the time-sequence of alterations taking place in course of function (VOGT and VOGT 1946, EDSTRÖM and EICHNER 1958, HYDEN 1959, PEVZNER 1964).

Attention was called to the special reaction of neurocytes by COHEN and JACKLET (1965) in course of their studies on the protein synthesis of the nerve cells in *Periplaneta americana*. They found that in case of axon-damages an RNA ring appeared in perinuclear localization in the regenerating nerve cell within 12 hours, and it became most marked on the 3—4th day. This phenomenon seems suitable to identify the cell body of the damaged fiber.

The nerves of the mussels are built up of unmyelinated fibers. Due to this the usual myelin-degeneration methods cannot be adopted to identify the nervous paths belonging to the single cell groups. The retrograde sign of regeneration, however, in case it does appear same as in the insects, might be of some help in localizing the neurocytes of axons passing in the different nerves.

The objective of the study presented here is primarily to elucidate the conditions related to the content and accumulation of nucleic acid in the nerve cells, because in connection with this we have no knowledge of systematic studies performed on Pelecypoda. Further, we wanted to establish, in general, whether the perinuclear RNA ring described above in *Periplaneta*, or some other signs of regeneration appear in the nervous system following intersection of the axons.

Material and method

The experiments were conducted on the cerebral, visceral and pedal ganglia of 14—16 cm long specimens of *Anodonta cygnea* L. Following excision from the animals the ganglia were fixed in Zenker solution for 3 hours. Oriented embedding of the various ganglia and attached small portion of tissue into celloidine-paraffine followed, and 8 μ sections were prepared.

Staining was made with methyl green-pyronine y (KURNICK 1955) and malachite green-pyronine y (PMaG) (BAKER and WILLIAMS 1965). Ribonuclease digestion (PEARSE 1961) was made to examine the RNA specificity of pyronine-staining.

Staining with the methyl green-pyronine y mixture proposed by KURNICK (1955) proved too pale, and, therefore, the stains were used differently from the original description, in the following ratio:

2 per cent pyronine y (GT, Gurr, England)	15.0 ml
2 per cent methyl green (NAD)	5.0 ml
Distilled water	20.0 ml

The PMaG staining method was applied with good result also without modification. This method was adopted later in the course of examinations, because the malachite green mixture can be preserved for a longer period without buffering.

The cleaning of the diluted solutions of the stains with $CHCl_3$ was made until no further changes were observable in the colour of the extracting chloroform. Evaporation of the purified stains followed and solutions of suitable concentrations were prepared again.

Stained sections were dehydrated with isobutyl alcohol, because, as observed, pyronine was more intensively dissolved by *n*-butyl alcohol than it was desired.

In digestion studies ribonuclease prepared of ox pancreas according to the method of BRACHET was used (GOMORI 1953).

In the course of light microscope evaluation of the sections comparative computations were made on basis of measurements with ocular-micrometer concerning the sizes of nuclei and nucleoli in cells that stained intensively or less intesively with pyronine.

Measurements on the length and width of nerve cells were made and on their basis cells of nearly equal sizes were selected. The cubic content of nuclei was calculated on basis of length and width measurements. In case the ratio of longitudinal and cross diameters of the nuclei was 1 : 1.2 the Fischer-Inke equation was used

$$V = \frac{\pi}{6}(\mathrm{LB}) \quad 3/2$$

(L = longer diameter, B = shorter diameter).

In case of nuclei that are more divergent from spherical shape computation was made according to PUFF's equation (PALKOVITS 1962);

$$V = \frac{8\,\pi\,F^2}{3L}$$

(F = surface, L = longer diameter.) Limit of error about 3 per cent.

In one group of molluscs the right side nervus pallialis posterior maior, in another groups the right branch of the cerebrovisceral connective (CVc) was intersected. Following operation the animals were slaughtered daily in 10—14 subsequent days and their cerebral, visceral and pedal ganglia were stained with PMaG and examined.

Results

All three pairs of ganglia of the central nervous system of *A. cygnea* exhibited a similar picture following staining with PMaG. The protrusions of the nerve and glia cells stained well with malachite green, whereas the cytoplasm, nucleus and the nuclei of the glia cells stained more palish. The nucleolus stained deeper with pyronine y, and the cytoplasm in the majority of the nerve cells and glia cells stained lighter with pyronine y.

The cytoplasm of some nerve cells in the cerebral and pedal ganglia stained intensively with pyronine y which was localized to a rough granular substance. Cells like these will be referred to later on as "pyroninophilous" cells. The whole cell volume is filled, in general, with these bright red granules *(Fig. 1)*. The nuclei of the pyroninophilous cells are small in comparison to

Fig. 1. "Pyroninophilous nerve cell" in the cerebral ganglion × 960

1. ábra. „Pyroninofil idegsejt" a ggl cerebraleban (960 ×)

those of other cells, and their shape is longitudinal. The nucleus is, in general, of central localization, but may also rather frequently be observable near the boundary of the cell. In most nuclei invaginations were observable. The nucleolus that stains with malachite green is extraordinarily small as compared to those of non-pyroninophilous cells and are, on occasion, scarcely visible even under immersion lens. The differences in volume between the nuclei of pyroninophilous nerve cells and of those of nerve cells of same dimension and staining normally are presented in *Table 1.*

The data show that the volume of the nuclei in pyroninophilous nerve cells are one third of that of the nuclei in nerve cells of similar size and staining less intensively with pyronine y.

Pyroninophilous nerve cells were observable chiefly in the cerebral and pedal ganglia. They numbered one or two in the sections but very often occurred only in the 4—5th subsequent sections each.

Nerve cells in which not the whole cytoplasm but only one or two bounded sections stain intensively with pyronine y are more seldom found. The area

Table 1

Volume of nuclei in pyroninophilous nerve cells and in normally stained ones

Pyroninofil és nem pyroninofil sejtek magvainak köbtartalma

	Number of cells	Size of nerve cells (μ)		Volume of nucleus (μ^3)
		length	width	
Pyroninophilous nerve cell	40	28.3 ± 3.8	21.0 ± 2.8	144 ± 48
Non-pyroninophilous nerve cell	40	28.2 ± 4.1	21.0 ± 2.0	560 ± 74

that stains intensively with pyronine y is most often localized near the origin of the axon or in the immediate neighbourhood of the nucleus. In case of partial pyroninophilia the nucleus is often of excentric localization. Nerve cells like these differ from the pyroninophilous cells in that their nuclei are, in general, round, their size is equal to that of nuclei in non-pyroninophilous cells and invaginations are not observable in them. Their nucleoli, same as those of non-pyroninophilous cells, are of 1—3 μ diameter, and stain intensively with pyronine y. These cells conform, however, to pyroninophilous cells in that the pyroninophilous area or areas are granular. The majority of such cells occurs, too, in the pedal and cerebral ganglia, and are to be found only seldom in the visceral ganglia.

The PMaG staining method is widely used for demonstrating RNA, but the specificity of pyroninophilia has to be established with other methods. The most suitable procedure serving this purpose is the digestion with ribonuclease. As we have no knowledge of similar studies performed on the nervous system of freshwater mussel, it was necessary to examine the relationship between pyroninophilia and RNA. For this purpose every second section of the series made of the same ganglion was incubated in ribonuclease and was stained with PMaG parallel with non-incubated sections. On basis of data obtained it was possible to establish the identity of the pyroninophilous substance with RNA, because the ribonuclease treated cells did not exhibit pyroninophilia.

Following the intersection of the pallial nerve and CVc the histological picture of the central nervous system is similar, in general, to the above ones, but besides this, special phenomena were also observable.

The first noteworthy phenomenon was that after 2—3 days following the intersection of the right n. pallialis or of the CVc a fine granular, homogeneously looking, ring-shaped zone staining intensively with pyronine, a so called "perinuclear ring" appeared around the nucleus of some cells in the visceral ganglion. This zone is best noticeable on the 3rd, 4th days, but is still observable also on the 7th and 8th days *(Fig. 2)*. The perinuclear ring is most marked in large sized and assumably motor cells. In nerve cells of small sizes the area that stained intensively with pyronine and surrounds the nucleus fills nearly the whole cytoplasm and therefore it is difficult to decide whether it is a perinuclear ring or perhaps the cytoplasm is stained slightly deeper than average. The nuclei of cells possessing a perinuclear ring are centrally localized. Their shape is elongated and no invaginations were observable in them. Their nucleoli which stain intensively with pyronine y are large (about 3—4 μ diameter). The cubic content of nuclei of nerve cells with perinuclear ring and of non-

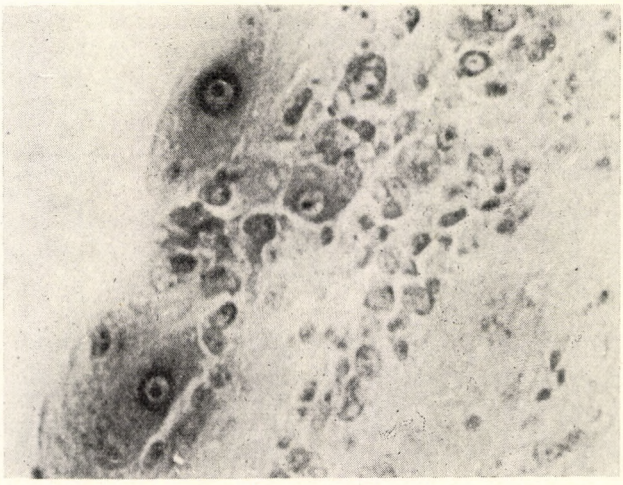

Fig. 2. Cells with perinuclear ring in the cerebral ganglion on the 3rd day following the intersection of CVc (× 960)

2. ábra. Perinuclearis gyűrűs sejtek CVc átmetszése után a 3. napon fixált ggl visceraleban (960 ×)

pyroninophil cells were calculated inside the same preparate. The values obtained are presented in *Table 2.*

According to the estimations the size of nucleoli in cells that possess a perinuclear ring is about three times as great as that of nucleoli in the control cells, whereas no difference in the volume of nuclei was observed.

In cerebral and pedal ganglia a typical perinuclear ring was not observable after the intersection of either n. pallialis or of CVc. The number of pyroninophilous perinuclear cells are few in the visceral ganglion, too, and they are dispersed. They may occur near the origin of chief nerve branches, in the nucleus posterior and in other areas of the cortex alike.

After digestion with ribonuclease the pyroninophilous perinuclear ring disappeares which indicates RNA.

The other noteworthy phenomenon observed was that in ganglia fixed on the 7th day following intersection of either pallial nerve or of CVc the glia cells stained deeper with pyronine y. The bright red colour of the glia cells surrounding the large sized nerve cells is most conspicuous *(Fig. 3).* Granulation is not noticeable under light microscope. Other glia cells are of deep

Table 2

	Number of cells	Size of nerve cells (μ)		Volume of nucleus (μ^3)
		length	width	
Nerve cells with perinuclear ring	20	33.4 ± 2.6	17.7 ± 2.1	596 ± 112
Non-pyroninophilous nerve cells	20	31.9 ± 2.8	18.3 ± 1.9	588 ± 77

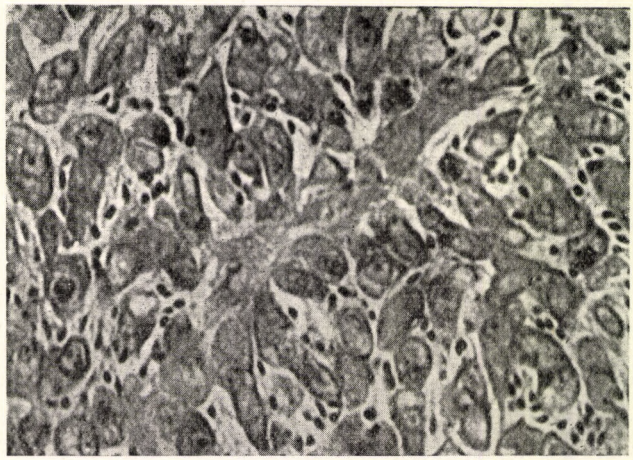

Fig. 3. Intensive pyroninophily of lamellar glia on the 7th day following intersection of the nerves (× 960)

3. ábra. Lemezes glia intenzív pyroninofiliája idegátmetszés utáni 7. napon (960 ×)

red colour and are granulated. In numerous glia cells granules which stained with malachite green were also observable. Glia cells staining intensively with pyronine y were observed in every three pair of ganglia.

The third characteristic feature observed was that on the 9th day following intersection of CVc glia fibers staining intensively with pyronine appeared in the visceral ganglion. This was not demonstrable either in cerebral or pedal ganglia. The majority of these fibres pass in the direction of the intersected CVc and leaves there the ganglion, and they are to be found at the origin of every chief nerve branch. Several fibers which stain with pyronine pass to the nerve cells of the cortex. After a more careful examination of the serial sections, it appeared, that the nerve cells of the cortex and chiefly those which are vacuolized are surrounded by the majority of pyroninophilous fibers. In other instances the pyroninophilous fiber terminates near the nerve cell in little club-shaped formations.

Parallel with the pyronine staining of the glia morphological alterations also occur in some nerve cells. On the 6—7th day following the intersection of pallial nerve one or two vacuoles appear in the cytoplasm of some nerve cells. The number of nerve cells containing vacuoles increases on the 8th—9th day and on the 10th day they may even amount to 50% of the total number of nerve cells. The vacuoles in some cells fill the whole cytoplasm and in such case the nucleus is shifted to the axon-hill or to the boundary of the cells. After the intersection of CVc the vacuoles appear sooner and in the glia examined on the 6th day vacuolized cells were predominating. Signs of desintegration appeared in about 3% of nerve cells on the 9th—10th day.

The size of the nuclei and nucleoli has changed parallel with the degree of vacuolization of nerve cells. At the beginning of vacuolization the nucleus is somewhat greater than in nerve cells of similar sizes without vacuolization. The size of nucleoli is above average and the nucleoli stain intensively with pyronine. The nuclei of nerve cells which contain large vacuoles are smaller,

they are elongated, and invaginations were also observable in them. In such nerve cells the nucleolus is smaller and stains less intensively with pyronine.

The average size of nuclei of vacuolized nerve cells does not differ from that of nuclei in the control nerve cells. It is to be noted, however, that in the size of nuclei great differences exist among vacuolized cells. Whereas in intact animals the quadratic deviation of the average volume of the nuclei (570 μ^3) was $\pm$ 75, the standard deviation of the average volume of nuclei (530 μ^3) of vacuolized cells of similar size was $\pm$212.

It is to be noted, finally, that the animals were alive for fourteen days following intersection of the right nervus pallialis posterior maior, whereas, after the intersection of the CVc only for maximum 10 days.

Discussion

Digestion studies with ribonuclease show that in that class of Mollusca which is examined in this study pyronine is specific to RNA and is, therefore, suitable for investigating the ribonucleic acid content and its alterations in nerve cells.

The cells of the ganglia of *Anodonta* contain, similar to other animals, different quantities of RNA. The cytoplasm and the nucleus of nerve cells stain, in general, similar to glia cells only weakly, in opposition to the nucleolus which stains mostly intensively. This staining is homogeneous which is indicative of the presence of finely distributed RNA. On another occasion, however, some regions of the cytoplasm and often the whole cytoplasm itself is extraordinarily rich in RNA which is present in the form of rough granules. Cells not containing RNA granules, and the partially or completely pyroninophilous cells are most obviously not characteristic of the type of the cells, but as it is known from literature (HYDEN 1960, BRODSKY 1966), of the rate of protein synthesis and might be related to the functional condition of cells. Parallel with changes in the periodic activity of freshwater mussel quantitative changes were demonstrable in the Nissl-substance (SALÁNKI, ZS.-NAGY and H.-VAS 1965) and in the nucleic acid content (ZS.-NAGY, BRODSKY and SALÁNKI 1966), the differences demonstrated in this study might also be connected with this.

In certain cells following intersection of the nerves the rich RNA content is different, it is not rough granular, and is localized in fine granular arrangement around the nucleus. This pattern might be related to the special condition of the cell, i.e. to regeneration.

It is probable that the rough granular RNA which may be localized in the cytoplasm of only partially pyroninophilous cells also at a greater distance from the nucleus is of different origin and is attached to different cell constituents than the fine granular, perinuclear RNA. In course of examining the changes in Nissl-substance both excessively granulated and homogeneously stained nerve cells were observable in the ganglia of *Anodonta cygnea* (SALÁNKI, ZS.-NAGY and H. VAS 1965). It is assumed that these two different structures might be identical to the so-called "compact" and "loose ergastoplasm" described previously in course of electronmicroscopic studies (DE ROBERTIS 1954), and might be explained likewise by the different origin of RNA.

The size of nuclei in pyroninophilous and non-pyroninophilous cells and in pyroninophilous cells with a perinuclear ring varied considerably. It is

known that at the beginning of increased synthesis the RNA content of the cells begins to increase in the nucleolus and that there is a correlation between nucleolar size and the RNA content of the cell (BERTRAM and BARR 1949, BRACHET 1955, EDSTRÖM and EICHNER 1958, STOWELL 1963). According to biochemical data chiefly transfer-RNA is to be found in the nucleolus (SIRLIN 1961, SIRLIN, KATO and JONES 1961) and thus the size of the nucleolus is indicative of the degree of protein synthesis. For this reason the difference in size observed among nucleoli of cells of equal sizes might be taken for the sign of the intensity of actual RNA and of related protein synthesis, respectively. The smallest nucleoli were observed in cells in which the cytoplasm was filled with RNA and it is assumed that in such cells transfer RNA production is most probably interrupted. In most cells the nucleoli are of medium size and this might perhaps be indicative of average protein synthesis.

The cells with pyroninophilous ring have extremely large nucleoli which is indicating very intensive RNA production and protein metabolism. This latter phenomenon is completely justified if the pyroninophilous ring is considered the sign of regeneration and also conforms to the data of VOGT and VOGT (1946) who observed in connection with "retrograde degeneration" the increase of nucleolus and the accumulation of the Nissl-substance.

In pyroninophilous cells not only the nucleolus is small but the nucleus, too, and on the nucleus invaginations are observable. This observation does not conform to the general opinion that the size of both nucleus and nucleolus is directly proportional to the RNA content of the cytoplasm, and can be explained so that in these cells the RNA-metabolism of both nucleus and nucleolus is restricted considerably due to the escessively great accumulation of nucleic acid and protein.

Cells with perinuclear RNA ring similar to those described by COHEN and JACKLET (1965) in the nervous system of *Periplaneta* were demonstrable in the visceral ganglia following the intersection of the nerves. The nuclei of these cells do not differ from the average but the size of the nucleoli is 3 times greater than average. It is undoubted that this might be related to increased protein synthesis and regeneration, respectively. At the same time two other phenomena were also observable following the intersection of nerves. On the 7th day the glia nuclei arranged around some nerve cells exhibited an intensive staining, and on the 9th day the glia protrusions that usually did not stain with pyronine turned pyroninophilous. It is known, that RNA was demonstrable also in glia cells and that its quantity is dependent on functional effects (HYDEN 1959, PEVZNER 1964). The glia has an important role in the food-supply of nerve cells and is, perhaps, important also in other respects, therefore, this phenomenon might be regarded as a sign of increased functioning. It cannot be neglected, however, that following the intersection of nerves and especially a week from intersection vacuolization is observed in some cells as a sign of degeneration. This cannot be explained by the alterations of the size of nuclei and nucleoli. Signs of degeneration were observed in other cells too, and it is not excluded that the pyroninophilia observed in glia cells, and on the protrusions might perhaps be related to degeneration and not to regeneration.

In the examinations presented in this paper, cells with perinuclear ring were observed only in the visceral ganglia following the intersection of either CVc or of pallial nerve. It is not clear why cells like these do not occur also in the cerebral ganglion, since the cell body of one part of fibers which pass in

the CVc are obviously inside the cerebral ganglion. An explanation to this might perhaps be found in the fact that in the large motor cells the perinuclear ring is most conspicuous and that the fibers of the motor cells of cerebral ganglia do not pass towards the visceral ganglion. Only studies in which the motor nerves will be intersected might give answer to these questions.

Summing up, it may be said that following axon damages a perinuclear pyroninophilous ring appears also in certain cells of Mollusca, and it is thought that this observation might be of use in localization studies. Further investigations are needed to elucidate the origin of pyroninophilia occurring in the glial cells and on the protrusions and to establish the relationship between their pyroninophilia and degeneration or regeneration.

It is not unlikely that the alterations observed in the glia are indicating the interrupted regenerative processes and the beginning of degeneration of deeply damaged nerve cells.

Summary

Applying malachite green pyronine staining on the ganglia of *Anodonta cygnea* the following observations were made:

1. Under normal conditions the cytoplasm of the nerve cells contains various amounts of RNA. In certain cells the cytoplasm is completely filled with RNA which is localized on rough granules ("pyroninophilous cells").

2. Both nuclei and nucleoli of the "pyroninophilous cells" are significantly smaller than those of non-pyroninophilous cells of similar size.

3. Following the intersection of nerves a fine granular perinuclear RNA appears in some cells. The nucleoli of these cells are about 3 times as great as those of control cells.

4. On the 7th day following nerve damages some groups of cells in the glia contain numerous RNA and on the 9th day RNA is demonstrable also in the protrusions of glia. At that time strong vacuolization is observable in some cells.

The perinuclear RNA ring is a regeneration phenomenon, and might be useful in localization studies. It is questionable, however, whether the strong increase of RNA content in the glia and vacuolization are related to regeneration or to degeneration processes of the nerve cells.

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RNS HISZTOKÉMIAI VIZSGÁLATA MOLLUSCA GANGLIONBAN NORMÁL VISZONYOK KÖZÖTT ÉS IDEGKÁROSODÁS UTÁN

Salánki János és *Gubicza András*

Összefoglalás

Malachitzöld-pyronin festés alkalmazásával *Anodonta cygnea* ganglionjában azt találtuk, hogy

1. Normál körülmények között az idegsejtek cytoplazmája különböző mennyiségben tartalmaz RNS-t. Vannak sejtek, melyek cytoplazmáját durva szemcsékre lokalizálódva teljesen kitölti („pyroninofil sejtek").
2. A „pyroninofil sejtek" magja és nucleolusa szignifikánsan kisebb, mint a hasonló méretű, nem pyroninofil sejteké.
3. Idegátmetszés után egyes sejtekben finomszemcsés perinuclearis RNS-gyűrű jelenik meg. E sejtek nucleolusa hasonló méretű kontroll sejt nucleolusánál kb. 3× nagyobb.
4. Idegkárosodást követő 7. napon a glia egyes sejtcsoportjai sok RNS-t tartalmaznak, a 9. napon RNS glianyúlványokban is kimutatható. Ebben az időben egyes sejtekben erős vakuolizáció észlelhető.

A perinuclearis RNS-gyűrű regenerációs jelenség, és lokalizációs vizsgálatokra alkalmas lehet. Kérdéses, hogy a glia RNS tartalmának feltűnő megszaporodása és a vakuolizáció is a regenerációval vagy pedig egyes sejtek degenerációjával függ-e össze.

ГИСТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ РНК В ГАНГЛИЯХ МОЛЛЮСКОВ ПРИ НОРМАЛЬНЫХ УСЛОВИЯХ И ПОСЛЕ НАРУШЕНИЯ НЕРВА

Янош Шаланки и *Андраш Губица*

При применении пиронина малахитовой зелени в ганглиях беззубки было обнаружено, что

1. — В нормальных условиях цитоплазма нервных клеток содержит разное количество РНК. Обнаруживаются такие клетки, цитоплазма которых полностью наполнена грубыми зернами («пиронинофильные клетки»).

2. — Ядро и ядрышко пиронинофильных клеток сигнификантно меньше чем в непиронинофильных клетках.

3. — После перерезки нерва в определенных клетках появляется околоядерное кольцо РНК, состоящее из мелких зерен. Ядрышко этих клеток в 3 раза больше, чем в нормальных клетках.

4. — Через 7 дней после перерезки нерва в отдельных клетках глии обнаруживается высокое содержание РНК, а через 9 дней уже и в отростках глии. В это время характерна и вакуолизация.

Околоядерное кольцо РНК является регенеративным явлением, и можно применять для локализации. Останется невыясненным вопрос о том, что наблюдаемое увеличение РНК в глии и вакуолизация регенерацией связаны ли или являются результатом дегенерации отдельных клеток.