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Histochemical Studies on the Distribution of Thiamine Pyrophosphatase and Enzymes Related to Carbohydrate Metabolism in the Intercalated Neurons of the Rat Supraoptic Nucleus

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ABSTRACT Histochemical studies have been conducted by applying hexokinase (HK), aldolase (AD), glyceraldehyde-3-phosphate dehydrogenase (G3), succinate dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G6PD), and thiamine pyrophosphatase (TPPase) methods, as well as Nissl staining and Gomori's chrome-alum-hematoxylin-phloxine (CHP) methods to intercalated neurons of the supraoptic nucleus (SO) on Wistar strain rats. Intercalated neurons reacted weakly to the AD, G3, G6PD, and SDH tests, indicating that they belong to the category of ordinary neurons with low carbohydrate metabolism. Many fibrous astrocytes showing strong HK reactions surround neurosecretory neurons. However, they do not surround intercalated neurons with mild HK activity. These results indicate that the latter receive a poor supply of energy from glucose in the circulating blood in contrast to the former. Intercalated neurons are very rich in Nissl substance but lack CHP-positive material. They may have a high potential for synthesizing protein. The principal morphological features of the TPPase-positive Golgi material are peculiar and heterogeneous shape and poor development. These findings together with mild G6PD activity suggest that intercalated neurons are very likely to have poor synthesizing activity.

Recently, certain studies that have approached the question in various ways have shown clearly the presence of a few small intercalated neurons in the supraoptic nucleus (SO) of some mammals.

Some electrophysiological studies have indicated the presence of inhibitory intercalated neurons ("neurosecretory Renshaw cells") in the SO of the rhesus monkey, dog, and cat (Vincent and Hayward, 1970; Koizumi and Yamashita, 1972). Léránth et al. (1975) reported that two-thirds of axon terminals are intranuclear in origin and that there is a population of small neurons in the SO that does not respond to osmotic stress. Furthermore, an immunohistofluorescence study (Swaab et al., 1975) indicated the presence of SO neurons that do not react to the antibodies of vasopressin and oxytocin. It is estimated that the more than 10% of SO neurons are nonsecretory.

Recent morphological studies with the Golgi-Cox method have clearly shown the presence of small multipolar neurons in the SO of rats and rabbits, which are considered to be intercalated neurons and are distinctly different from neurosecretory neurons (Felton and Cashner, 1979; Dyball and Kemplay, 1982).

In spite of these studies, the histochemical characteristics of these intercalated neurons are not as yet known well. Although there are some doubts concerning the reliability of the thiamine pyrophosphatase (TPPase) method as a marker of the Golgi apparatus (GA), it seems to be still useful for comparing the main features of the GA of two kinds of neurons in the same section (cf. Shanthaveerappa and Bourne, 1965 a-d; Shantha and Bourne, 1966 a,b; Luppa et al., 1979).

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The present investigation of intercalated neurons was made to study the pattern of carbohydrate metabolism, energy supply, and the main features of GA morphology, by applying the hexokinase (HK), aldolase (AD), glyceraldehyde-3-phosphate dehydrogenase (G3), succinate dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G6PD), and thiamine pyrophosphatase (TPPase) methods on the rat SO. In addition, the presence or absence of Nissl substance and neurosecretory material was verified in order to distinguish neurosecretory neurons and intercalated neurons. Karyometry and statistical procedures were applied to confirm the accuracy of our identification method for two kinds of neurons and glial cells.

MATERIALS AND METHODS

Twenty-five healthy adult male rats of the Wistar strain (body weight of 200–350gm) were used in this study.

Enzyme-histochemical procedures

A thin slice of the hypothalamus, including the supraoptic nucleus, was cut immediately after decapitation of the animals without anesthesia. The slice was frozen in dry-ice (−55°C), placed on the cryostat chuck, and sectioned at 10 μm at −20°C with a Pearse-Slee cryostat. The following methods were used to test the enzymes: hexokinase by the Meijer (1967) method (incubation period 25 min), aldolase by the Abe and Shimizu (1964) method (60 min), glyceraldehyde-3-phosphate dehydrogenase by the Fasske and Steins (1963) method (20 min), succinate dehydrogenase by the Nachlas et al. (1957) method (60 min), glucose-6-phosphate dehydrogenase by the Negi and Stephens (1977) method (20 min), and thiamine pyrophosphatase by the Novikoff and Goldfischer (1961) method (20 min).

Demonstration of Nissl substance and neurosecretory material

A part of the hypothalamus, including the SO, was fixed by immersion in 10% formalin or in Zenker's fluid and was sectioned at 8 μm after being embedded in paraffin. The sections fixed in formalin were stained by the Klüver and Barrera (1953) method for the verification of Nissl substance. Gomori's (1941) chrome-alum-hematoxylin and phloxine (CHP) method (1941) was applied to the sections fixed in Zenker's fluid in order to

Abbreviations

A	Arachnoidal lining
B	Blood vessel
iN	Intercalated neuron
n	Nucleolus
N	Neurosecretory neuron
Np	Neuropil
Nu	Nucleus
PZ	Perinuclear zone just dorsal to the SO
S	Subarachnoid space
SO	Supraoptic nucleus

Fig. 1. Nissl substance is particularly dense in the perikarya of intercalated neurons (thick arrows) in comparison to neurosecretory neurons, where Nissl substance is limited to the peripheral part of the perikarya (thin arrows). ×375.

Fig. 2. The perikarya of neurosecretory neurons show many fine CHP-positive granules whereas an intercalated neuron and glial cells (unlabeled arrows) lack CHP-positive material. ×1,500.

Fig. 3. Part of the SO showing the hexokinase reaction at a high magnification. A neurosecretory neuron with moderate activity is surrounded by many glial cells (black and white unlabeled arrows) and their processes (p) with strong activity. The nucleus and nucleolus of an intercalated neuron show no reaction, but its cytoplasm shows mild activity. ×1,500.

Fig. 4. Low-power photomicrograph showing the hexokinase reaction in the area ventral to the SO. A blood vessel in the subarachnoid space and an arachnoidal lining show mild and strong activity, respectively. A layer of many astrocytes showing several groups (surrounded by arrows) exhibits very strong activity. ×375.

Fig. 5. Part of the SO showing the aldolase reaction. Both nuclei of a neurosecretory neuron and an intercalated neuron show no reaction. The perikaryon of the neurosecretory neuron shows strong activity, whereas that of the intercalated neuron exhibits moderate activity. A glial cell (unlabeled arrow) shows mild activity. The neuropil reveals scattered, mildly to moderately positive granules. ×1,500.

Fig. 6. Part of the SO showing the glyceraldehyde-3-phosphate dehydrogenase reaction illustrated at a higher magnification than in Figure 5. The nucleus and nucleolus of the neurosecretory neuron, and the nucleus of the intercalated neuron show no reaction. The perikarya of both neurons show moderate activity. A glial cell (unlabeled arrow) shows a mild reaction, whereas the neuropil exhibits negligible activity. ×2,190.

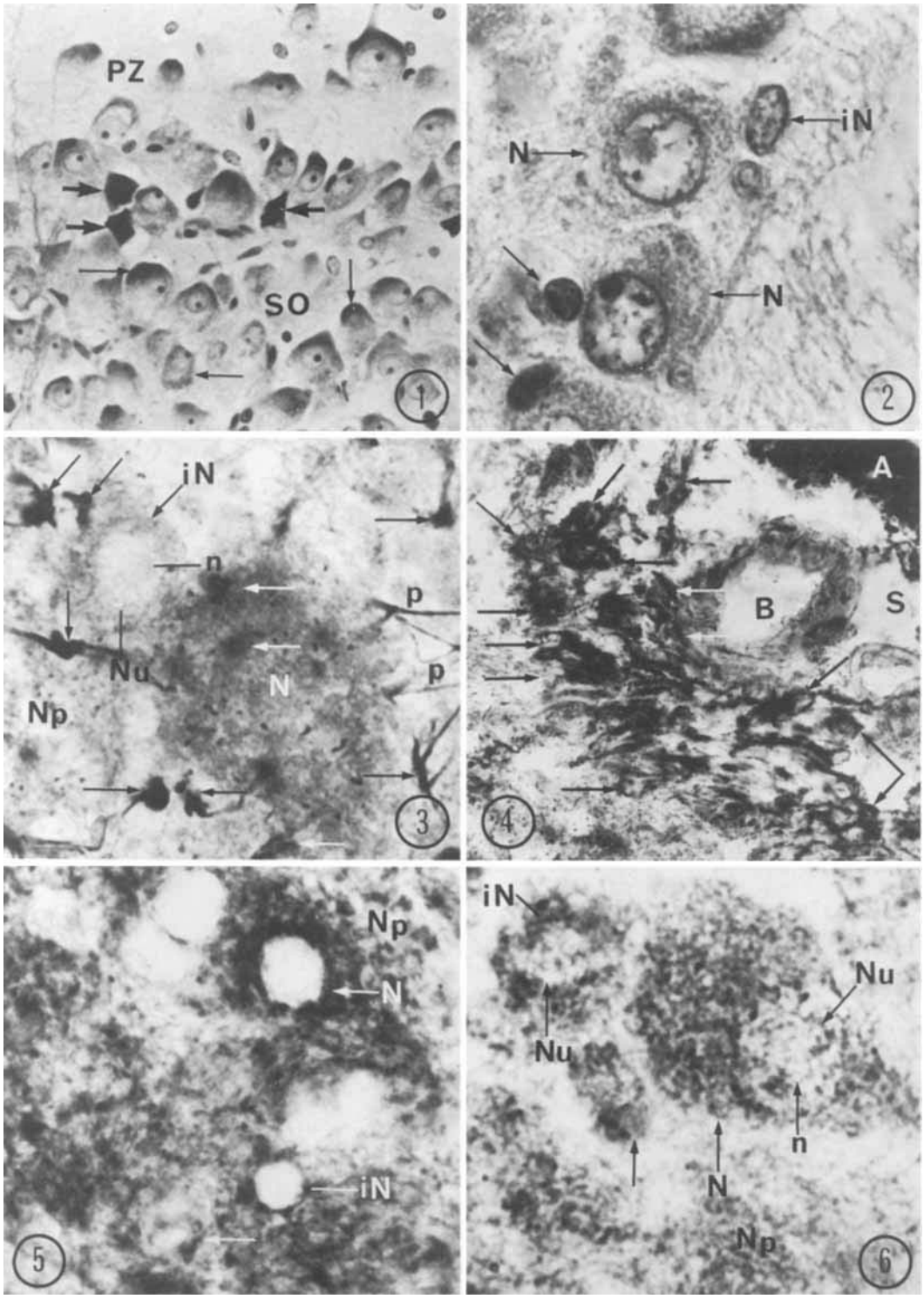
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TABLE 1. Reactions of some important components of the supraoptic nucleus to various histochemical tests

Reactions	Intercalated neurons			Neurosecretory neurons			Glial cells	Neuropil
	Nucleolus	Nucleus	Cytoplasm	Nucleolus	Nucleus	Cytoplasm		
Nissl	–	–	+++	–	–	+	±	–
CHP	–	–	–	–	–	– ~ +++	–	–
HK	–	–	+	–	–	++ > +++	+++ ~ ++++	±
AD	–	–	+ ~ ++ > +++	–	–	+ ~ +++	+	+
G3	–	–	+ > ++	–	–	+ > ++	± ~ +	±
SDH	–	–	+ > ++	–	–	+ ~ ++ ±	±	+
G6PD	–	–	+ >> ++	–	–	+ ~ +++	+++	+ ~ ++

Abbreviations: AD, Aldolase; CHP, Gomori's chrome-alum-hematoxylin and phloxine; G3, glyceraldehyde-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; HK, hexokinase; Nissl, Nissl substance; SDH, succinate dehydrogenase. Types of reactions: ~ = negative, ± = negligible, + = mild positive, ++ = moderate positive, ++ ± = moderately strong positive, +++ = strong positive.

stain the neurosecretory material selectively.

Statistical procedures

In order to confirm the certainty of our identification method for neurosecretory neurons, intercalated neurons, and glial cells, karyometry was done on Nissl preparations of the SO by using a phase-contrast microscope (Nikon, DLL × 100) and a screw-micrometer eyepiece (Nikon). The eyepiece allowed a margin of error of less than 0.1 μm. Both the longest (A) and shortest (B) axes of the nuclei of all cells existing in more than five SO areas were measured. Five hundred and nineteen nuclei were measured in this study. Nuclear volumes were calculated according to the formula

$$V = 4\pi/3\,(A/2)\,(B/2)^2$$

The t test was used to check the differences in nuclear volume between any two of the three cell groups.

RESULTS

Nissl substance

This substance was denser in the perikarya of intercalated neurons, with small irregular, multipolar shapes, than in neurosecretory neurons with large fusiform shapes, where its distribution was limited to the peripheral part of the cytoplasm (Fig. 1, Table 1).

The mean nuclear volume (M ± SD) of neurosecretory neurons, intercalated neurons, and glial cells was 333.53 ± 71.63 μm^3 (n = 266), 105.38 ± 44.68 μm^3 (n = 46), and 80.66 ± 28.14 μm^3 (n = 207), respectively. The results of the t test indicate that differences in nuclear volume between any two of the three cell groups were significant ($P < 0.005$).

Neurosecretory material

Intercalated neurons lacked this material (Fig. 2, Table 1). Glial cells did not reveal any CHP-positive material, and they were small (Fig. 2). However, they could be distinguished from intercalated neurons by comparing their nuclei with nuclei of glial cells in the optic tract.

Hexokinase (HK)

Some neurosecretory neurons showed strong activity in their perikarya, while the others had only a moderate reaction (Fig. 3, Table 1). The nuclei and nucleoli of all neurons, including intercalated neurons (Fig. 3), showed no reaction. It should be noted that the perikarya of all intercalated neurons demonstrated only mild activity (Fig. 3, Table 1). The neuropil of the SO had a negligible reaction.

Glial cells, including their straight processes, manifested very strong activity. They aggregated in the SO more densely than in other areas, where each neurosecretory neuron was surrounded by many glial cells (Fig. 3). It should be noted that every intercalated neuron showing a mild reaction (Table 1) was isolated from glial cells and blood vessels (Fig. 3), which was quite different from the case of a neurosecretory neuron.

A layer of glial cells was observed ventral to the SO. In the area ventrolateral to the SO, a layer was found consisting of groups of astrocytes displaying strong activity (Fig. 4). It was noted that some of these glial cells extend processes slightly toward the SO.

Aldolase

The nucleus and nucleolus of the intercalated neuron did not exhibit any reaction, while its perikaryon showed variable reactions (Table 1). As a result of our experiment,

in most cases the perikaryon manifested mild to moderate activity (Fig. 5), but in the remaining few cases there was a strong reaction. In general, intercalated neurons had less activity than neurosecretory neurons (Fig. 5, Table 1). In these two kinds of neurons, the positive reaction was both diffuse and granular. Glial cells exhibited mild activity. The neuropil showed scattered, fine and coarse positive granules (Fig. 5, Table 1).

Glyceraldehyde-3-phosphate dehydrogenase

Every component of the SO displayed significantly less activity to this test than to the AD test (Table 1). Most intercalated and neurosecretory neurons reacted to the G3 test by exhibiting mild activity in their perikarya, whereas the remaining neurons had only moderate reactions (Fig. 6). Glial cells displayed negligible to mild activity (Fig. 6). The neuropil was difficult to observe.

Succinate dehydrogenase

Most intercalated neurons manifested mild activity whereas the others had only moderate reactions (Table 1). The activity was both diffuse and granular (Fig. 7). Neurosecretory neurons with variable reactions appeared to have a higher level of activity than intercalated neurons (Fig. 7, Table 1). Glial cells had negligible activity, and the neuropil exhibited mild reactions (Fig. 7).

Glucose-6-phosphate dehydrogenase

Almost all intercalated neurons had mild reactions in the cytoplasm; only a few exhibited moderate activity (Fig. 8, Table 1). In contrast to intercalated neurons, neurosecretory neurons varied markedly in the intensity of the reaction (Table 1). Some of these neurosecretory neurons exhibited strong, diffuse activity in the cytoplasm, whereas others had only a mild to moderate reaction (Fig. 8).

It should be noted that neurosecretory neurons with mild activity were observed; these were in marked contrast to other strongly positive neurosecretory neurons in the same nucleus. The existence of these mildly positive neurons should not be overlooked (Table 1).

The neuropil had mild to moderate activity. All glial cells had strong reactions (Fig. 8).

Thiamine pyrophosphatase (TPPase)

The following descriptions are limited to the results of intercalated neurons only, because descriptions of neurosecretory neurons and glial cells in the rat were previously published (Iijima, 1977, 1979).

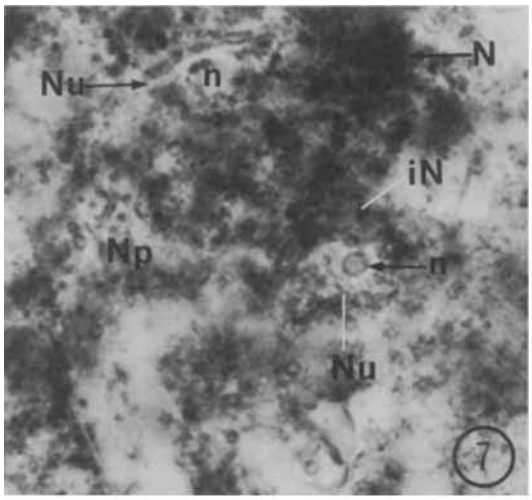
After 20 min of incubation the individual neurons manifested different types of positive reactions (Figs. 9–12). The nucleus and nucleolus were negative (Figs. 10,12). The cytoplasm, except for the GA, was unstained (Figs. 9–12). Generally, the GA is peculiar and heterogeneous in shape, with poor development in comparison to neurosecretory neurons (cf. Iijima, 1979). The phenomena of disintegration and the budding-off process (Shanthaveerappa and Bourne, 1965d; Iijima, 1970) could not be observed.

In a few neurons (Fig. 9), the GA was composed of fine and coarse granules usually of the same size. They were scattered randomly in the cytoplasm. The strands interconnecting them are difficult to observe. However, a thin, short strand consisting of very fine separate granules was observed in rare cases (Fig. 9). In a few other neurons, the GA was divided into separate portions that had a peculiar appearance. Heterogeneous shapes were prominent in these cells (Fig. 10). In some neurons, a network of the GA was peculiarly shaped and connected with various elements such as granules, vesicles, loops, clusters of granules, and small reticular networks with granules (Fig. 11). The GA of a few neurons had a diffusely stained, star-like multipolar appearance. The nucleolus did not display a reaction (Fig. 12).

DISCUSSION

When tests were made on AD, G3, and SDH, the neurosecretory neurons manifested almost equally mild activity, whereas variably mild to strong reactions were observed on the G6PD test in this study (Table 1). Intercalated neurons, on the contrary, exhibited the same degree of mild reaction to all the enzyme-histochemical reactions including G6PD (Table 1).

Previous studies on the neurosecretory neurons on the rat, mouse, rabbit, and guinea pig (Shimizu et al., 1957; Shimizu and Morikawa, 1957; Friede et al., 1963; Friede, 1966) concluded that these neurons should be placed in the category of "exceptional nuclei" such as the locus coeruleus and dorsal vagal nucleus in regard to the pattern of carbohydrate metabolism; the neurons are poor in SDH and cytochrome oxidase in contrast to their richness in G6PD and lactate dehydrogenase (Friede, 1966). The conclusion that the neurosecretory neurons should be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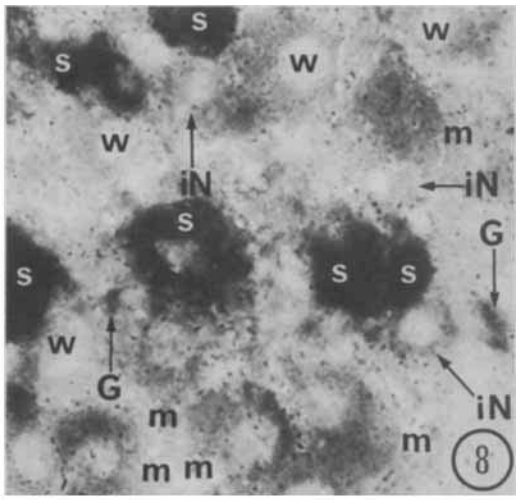
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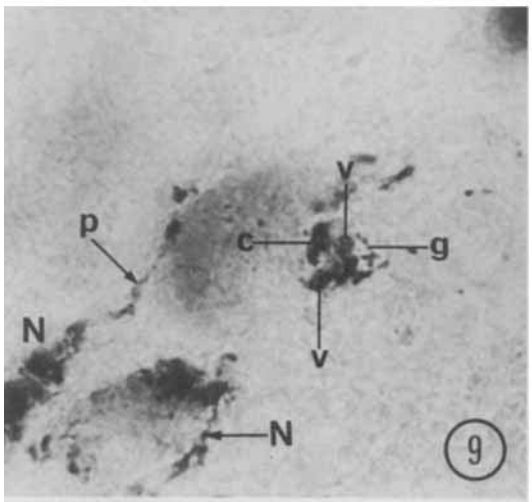
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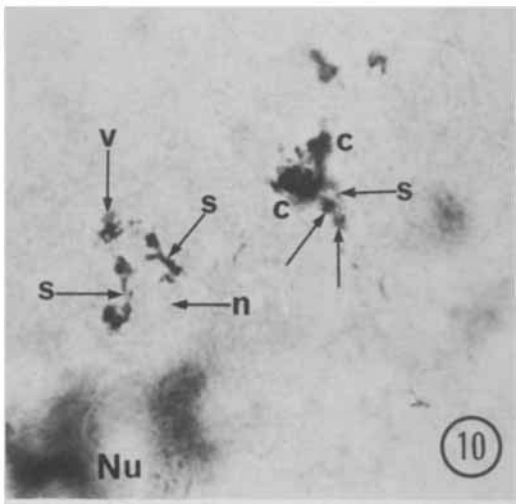
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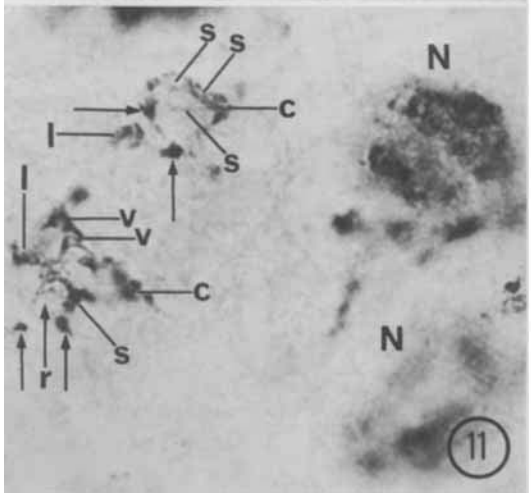
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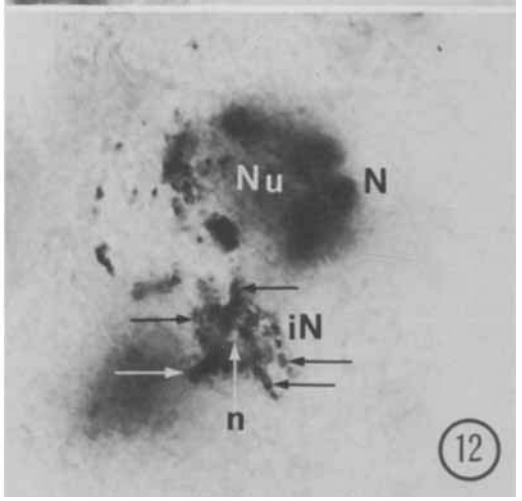
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Abbreviations

c	Cluster of granules	n	Nucleolus
g	Fine granules	Nu	Nucleus
iN	Intercalated neuron	v	Vesicle
N	Neurosecretory neuron		

Fig. 7. Part of the SO showing the succinate dehydrogenase reaction at a high magnification. The nucleus and nucleolus of both the neurosecretory neuron and the intercalated neuron show no reaction. The perikaryon of the intercalated neuron shows moderate activity, whereas that of the neurosecretory neuron shows moderately strong activity. The neuropil shows mild activity. ×1,500.

Fig. 8. Part of the SO showing the glucose-6-phosphate dehydrogenase reaction. Two intercalated neurons show moderate activity in their perikarya (upper left and lower right), whereas one intercalated neuron shows mild activity (upper right). Neurosecretory neurons show variable reactions. Six neurons showing strong activity (s), 5 neurons showing moderate activity (m), and 4 neurons showing mild activity (w), are recognizable in this figure. Glial cells (G) show strong activity. ×600.

Figs. 9–12. Intercalated neurons showing the thiamine pyrophosphatase (TPPase) reaction illustrated at a high magnification. ×1,500.

Fig. 9. Vesicles and a cluster of granules gather together. In addition, a thin strand consisting of fine granules is observed. In the lower left corner, the TPPase-positive Golgi material (GA) of two neurosecretory neurons is visible. The neuron at left shows a GA extension in a process (p). ×1,500.

Fig. 10. The GA of both intercalated neurons in the center area is highly peculiar in shape. The GA on the left side is made of three separate portions. One portion is a cluster of fine vesicles, and the other two parts show a similar structure consisting of a short strand (s) connected with granules on both ends. On the right, there is a Y-shaped GA. A thin strand (s) makes an irregular network connected with granules (arrows) and clusters of granules. In the lower left corner, a neurosecretory neuron exists, where the nucleus is observed. ×1,500.

Fig. 11. The GA of both intercalated neurons is a network peculiar in shape. In the upper left corner, the GA is composed of thin and thick strands (s) connected with granules (arrows), a loop (l), and a cluster of granules. In the lower left corner, the GA network is peculiar in the variety of its components as well as in shape. It consists of strands connected with granules (arrows), vesicles, a loop (l), a cluster of granules, and a localized reticular network including fine granules (r). On the right side of this figure, two neurosecretory neurons show the TPPase-positive reaction. ×1,500.

Fig. 12. The nucleus of a neurosecretory neuron shows no reaction. In the lower center area, the GA of an intercalated neuron reveals several TPPase-positive processes (arrows) and a star-like multipolar appearance. The perikaryon shows moderate activity. The nucleolus exhibits no reaction. ×1,500.

classified as exceptional nuclei (Shimizu et al., 1957) is questionable, as previously pointed out by Iijima et al. (1967), when our present findings that a few neurosecretory neurons show mild G6PD activity are taken into consideration.

Intercalated neurons similarly equipped with the enzymes of the Embden-Meyerhof (EM) pathway, the Warburg-Dickens pathway (HMP shunt), and the tricarboxylic acid cycle (TCA cycle) should be categorized as "ordinary nuclei" as far as the pattern of carbohydrate metabolism is concerned (Iijima and Imai, 1975). But these neurons are different from usual "ordinary nuclei," such as the hypoglossal nucleus and the mesencephalic tract nucleus of the trigeminal nerve, where the enzymes of these pathways show much greater activity than intercalated neurons. In this respect, intercalated neurons are very similar to those of the Edinger-Westphal nucleus of the rat (Iijima and Imai, 1976). It seems logical to conclude that intercalated neurons belong to the "ordinary nuclei" with a low level of carbohydrate metabolism (Friede, 1966).

It is known that G6PD is closely related to a secretory function (Abe et al., 1963) and that G6PD activity parallels the development of the GA in the rat neurosecretory neurons (Iijima, 1979) which produce neurosecretory substances (Sano and Knoop, 1959; Palay, 1960; Osinchak, 1964). Mild G6PD activity shown by intercalated neurons in the present study indicates that they are nonsecretory and are not directly related to hormone production as cited in Swaab et al. (1975).

It seems important to notice that the patterns of energy supply are significantly different between neurosecretory and intercalated neurons. Biochemically, neurons receive their energy supply from glucose in the circulating blood (Holmes and Holmes, 1926; Takagaki, 1964). The rat SO is very rich in capillary beds (Craigie, 1940). HK-rich fibrous astrocytes (LuQui and Fox, 1976) closely surround each neurosecretory neuron that shows more than moderate HK activity. On the contrary, intercalated neurons with mild HK activity lack such glial supports. It is to be noted that these neurons get much less energy supply from glucose than neurosecretory neurons.

In addition, ventrolateral to the SO, a layer of groups of astrocytes showing strong HK activity has been found in the present study. We assume that they may play a role as an

energy donor to the SO by utilizing the diffused substances from the blood vessels in the subarachnoid space ventral to the brain surface.

Intercalated neurons were found to be very rich in Nissl substance and lacked CHP-positive material in this study. It is known that even neurosecretory neurons can become CHP-negative during a stage of phasic change (Zambrano and Mordoh, 1966). Furthermore, it was noted in this study that CHP-negative neurosecretory neurons are larger than intercalated neurons. Some authors reported that CHP-negative neurons were recognizable in the SO of rhesus monkeys and bats (Palay, 1953; Troyer, 1965). Troyer (1965) emphasized that CHP-negative neurons were much smaller than CHP-positive neurons. These smaller neurons should be considered to correspond with our intercalated neurons, and the negative neurons proven by the histofluorescence method (Swaab et al., 1975), and the multipolar neurons shown by the Golgi-Cox method in the rabbit and rat SO (Felton and Cashner, 1979; Dyball and Kemplay, 1982). Nissl substance is very active in protein synthesis (Nievel and Cumings, 1967). Therefore, the presence of abundant Nissl substance in intercalated neurons suggests strongly that they have a high potential for synthesizing protein.

The TPPase method cannot define the whole GA shape exactly, but it is still useful for visualizing its main features (Shanthaveerappa and Bourne, 1965a–d; Shantha and Bourne, 1966 a,b; Iijima, 1975). As shown by Figures 9–12, the TPPase-positive Golgi material of intercalated neurons is poorly developed. It is peculiar and heterogeneous in shape compared with that of neurosecretory neurons of the rat and rabbit (Iijima, 1970, 1979). The present findings, together with the poor G6PD activity shown by intercalated neurons (cf. Abe et al., 1963), suggest strongly that these neurons must have poor synthesizing capacity.

In summary, it seems logical to conclude—on the basis of low activity in the EM pathway, the HMP shunt and the TCA cycle; a poor energy supply to the intercalated neurons; poorly developed TPPase-positive Golgi material; and an abundance of Nissl substance—that intercalated neurons of the rat SO are ordinary neurons with regard to the pattern of carbohydrate metabolism. These neurons should be inactive in carbohydrate metabolism as well as in synthesizing activity. It is believed that intercalated neurons have a high potential for synthesizing protein owing to the presence of abundant Nissl substance.

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