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Granulated Vesicles in Retinal Synapses and Neurons*

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Summary. The electron microscope study of the retina of rats revealed the presence of granulated vesicles in synapses and neurons. Of the three different types of nerve endings observed in the inner plexiform layer, two were found to contain granulated vesicles. Type 1 nerve ending, belonging to bipolar cells, is devoid of granulated vesicles and together with type 2 nerve endings and ganglion cell dendrites forms glomerular-like synapses. Type 2 nerve endings, belonging to amacrine cells, have granulated vesicles of 640 Å, and type 3 nerve endings, probably representing centrifugal fibers and located at the outermost zone of the inner plexiform layer, have granulated vesicles of 1140 Å.

Granulated vesicles were also found in perikarya of the inner nuclear layer in close association with the Golgi complex of these cells. Of the two types of neurons observed in the ganglion cell layer, the clear type neuron exhibits granulated vesicles in its cytoplasm.

The correspondence of these findings with those obtained with the histochemical fluorescence technique for catecholamines is discussed. The suggestion is made that the granulated vesicles could represent the site of storage of retinal catecholamines.

According to CAJAL (1904), the synaptic pattern of the inner plexiform layer of the retina is formed by the interconnection of three neurons, i.e. bipolar, ganglion cells and amacrines, with the addition of the centrifugal fibers of central origin. With these components two neuronal chains may be formed: a) a centripetal chain comprising: photoreceptors, bipolars and ganglion cells; b) a centrifugal chain involving centrifugal fibers, amacrine and ganglion cells. CAJAL described the centrifugal fibers as ending in contact with perikarya and processes of the amacrine cells and recognized several synaptic layers containing the interconnected processes of the amacrines, cone-bipolars and ganglion cells. Rod-bipolars were described as making direct contact with the perikarya of ganglion cells.

The electron microscope study of DOWLING and BOYCOTT (1965) lead to the interpretation that the amacrine cells form a complex horizontal system having presynaptic contacts on bipolar terminals. They described the so-called "dyads" as the site at which a bipolar cell contacts with two postsynaptic elements represented by the amacrine and ganglion cells. They postulated the existence of "reciprocal synapses" between the amacrines and bipolars at which nerve transmission could travel both ways.

Using the fluorescence method for the study of catecholamines, MALMFORS (1963) found an intense yellow-green fluorescence at the outermost limit of the inner plexiform layer which was interpreted as due to dopamine (HÄGGENDAL and

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MALMFORS, 1963). Although no electronmicrographs were published by the Swedish authors they mention the finding of granulated vesicles in nerve cell processes and in few nerve cell bodies of that region.

The present investigation was initiated with the idea of learning about the exact localization of the granulated vesicles within the complex synaptic pattern found in the inner plexiform layer of the retina. We will describe three different types of nerve endings two of which contain granulated vesicles. In addition the finding of similar vesicles in perikarya of the inner nuclear and ganglion cell layers will be reported. A preliminary paper in abstract form has already appeared (PELLEGRINO DE IRALDI and JAIM ETCHEVERRY, 1967a).

Material and Methods

Retinas from male albino rats (150—200 g), killed by decapitation during day time were quickly dissected and inmersed in a solution of 3% glutaraldehyde (purified in Amberlite CG 50 according to Zieher, personal communication) in phosphate buffer 0.1 M, pH 7.3 (SABATINI et al., 1963). The fixation was carried at 0—4° C for various periods of time. After washing the specimens in 0.3 M sucrose in the same buffer overnight they were post-fixed in 1.5% buffered osmium tetroxide at 0—4° C for 90—120 min. Prior to dehydration, the pieces were inmersed in 2% aqueous uranyl acetate for 150 min and then dehydrated in graded increments of ethanol, being finally embedded in Epon 812 (LUFT, 1961). Thin sections were obtained in an Ultrotome LKB and mounted on naked copper grids. After staining with lead citrate (REYNOLDS, 1963) they were examined in a Siemens Elmiskop I.

Observations

Granulated vesicles in nerve endings

Three different types of nerve endings may be identified within the inner plexiform layer of the rat retina by their general morphology and vesicular content.

a) Type 1 ending is found at all levels of the layer and represented by terminals of varying size, the largest of which having a diameter of 7 μ are located in the vicinity of the ganglion cell layer (Fig. 1). These nerve endings are characterized by numerous and evenly distributed clear vesicles of about 400 Å, most of which are round shaped and some elongated. Sections of neurotubules (DE ROBERTIS and FRANCHI, 1953) are often seen intermingled with the synaptic vesicles. Some mitochondria and a few large vesicles of the endoplasmic reticulum may be observed with more abundance at the pedicle of the ending. Small synaptic ribbons similar to those found in the receptor cell terminals by DE ROBERTIS and FRANCHI (1956) and others may be observed.

Type 1 endings have been previously described by KIDD (1962) and DOWLING and BOYCOTT (1965) and interpreted as belonging to bipolar cells. In most aspects

Fig. 1. Retinal glomerulus is formed by a type 1 nerve ending, belonging to a bipolar cell, two type 2 terminals of the amacrine cells and several ganglion cell dendrites. In type 1 nerve ending there are clear vesicles, neurotubules and mitochondria. In type 2 terminal clear and small granulated vesicle (arrow), as well as some mitochondria may be observed. × 60000
All figures are electronmicrographs of rat retina fixed in glutaraldehyde, post-fixed in osmium tetroxide, and stained with uranyl acetate and lead citrate.
Abbreviation for all figures. a active points in synapses; *AC* amacrine cell; *d* ganglion cell dendrite; *G* Golgi complex; *Ly* lysosome; *mi* mitochondria; *N* nucleus; N_1 type 1 nerve ending; N_2 type 2 nerve ending; N_3 type 3 nerve ending; *nt* neurotubule

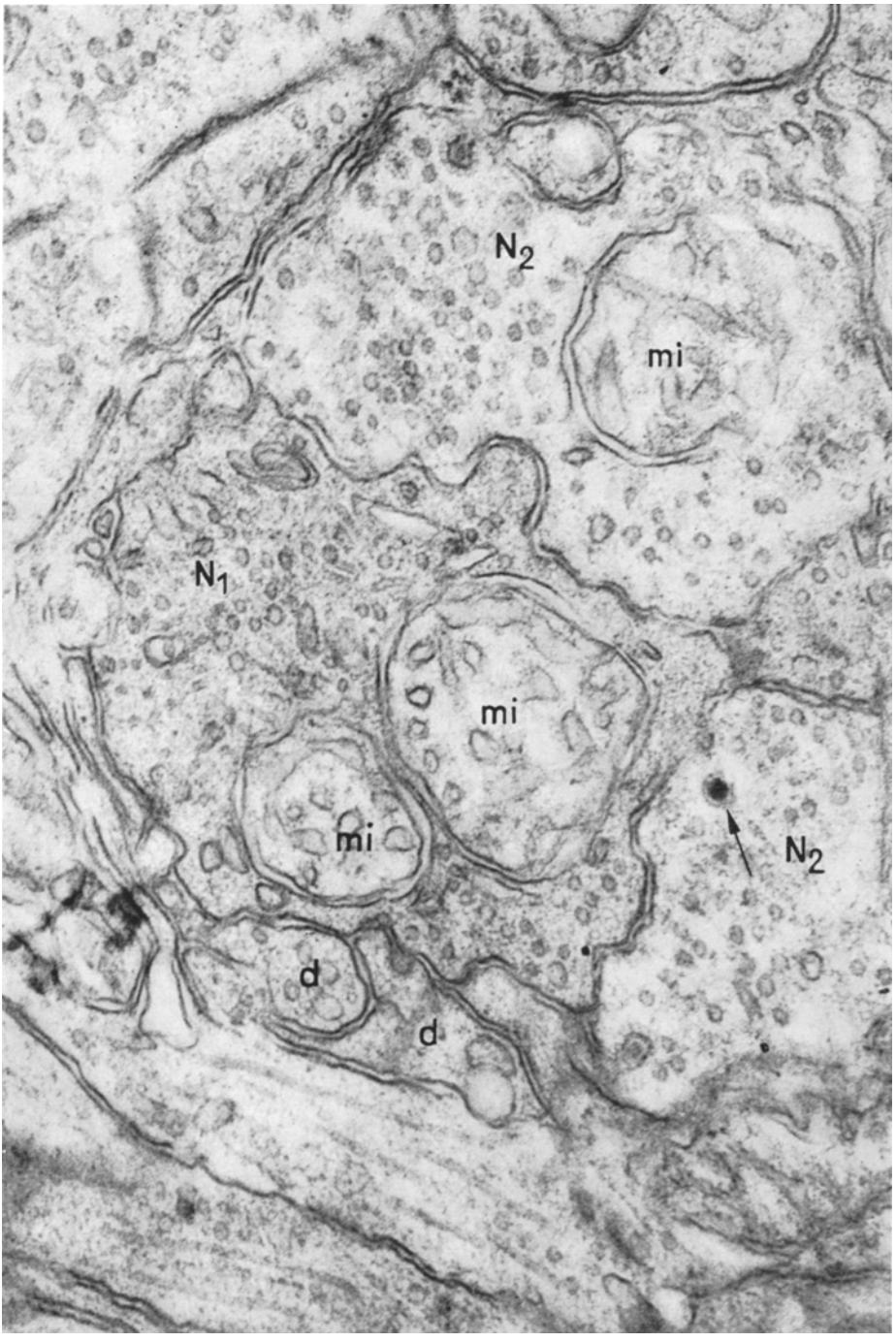

Fig. 1 (for legends see p. 284)

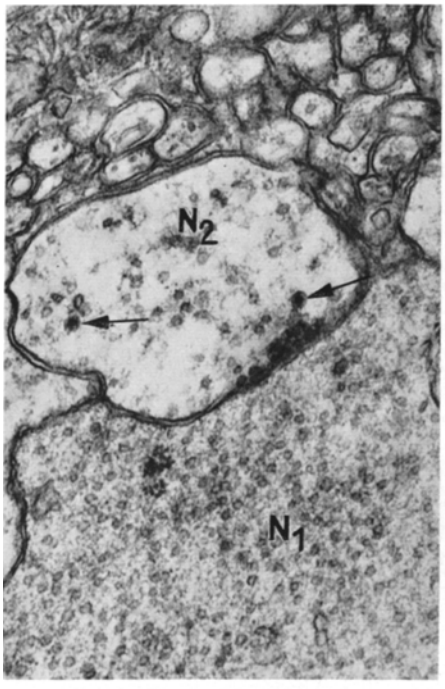

Fig. 2

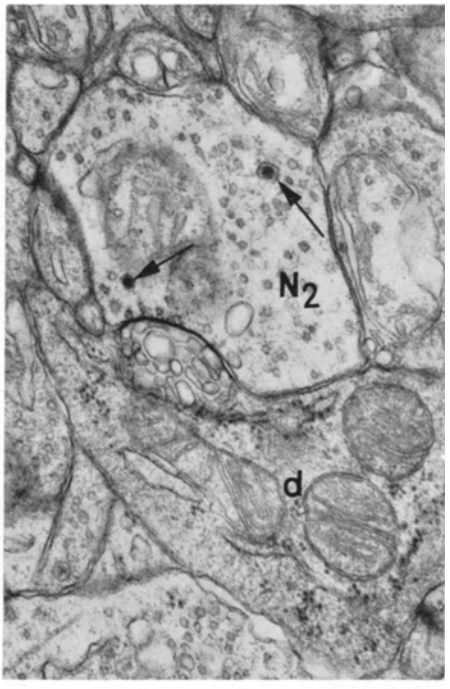

Fig. 3

Fig. 2. Type 2 nerve ending of an amacrine cell with clear and small granulated vesicles (arrows) making contact on a type 1 nerve ending of a bipolar cell. × 26000

Fig. 3. Nerve ending type 2 of an amacrine cell in contact with a ganglion cell dendrite. In the terminal two small granulated vesicles (arrows) and numerous clear vesicles, some of them attached to the presynaptic membrane, may be seen. Large vesicles are observed in the dendrite under the subsynaptic membrane. × 31000

our findings are very similar but they differ in some details. The bipolar ending makes contact with two postsynaptic elements: type 2 endings of the amacrine cells and dendrites of the ganglion cells. This complex forms the so-called dyad of DOWLING and BOYCOTT (1965). In addition amacrine cell processes (type 2 endings) appear to make presynaptic connections on the bipolar terminals as indicated by the accumulation of vesicles on the synaptic membrane (Fig. 2). Such contact may be isolated or may form a more complex glomerular structure in which type 1 ending is surrounded by type 2 and by ganglion cell dendrites (Fig. 1). The large type 1 endings from rod-bipolars, may be observed to make direct contact with the perikarya of ganglion cells.

b) Type 2 ending is also present at all levels of the layer (Figs. 1, 2 and 3). Its size is much smaller, not exceeding 1.7 μ, and the content less dense than type 1. These endings contain mitochondria but do not have synaptic ribbons. The vesicular content is mixed, with clear vesicles of about 400 Å and granulated vesicles having a mean size of 640 Å (range between 550—1600 Å). These vesicles have a dense core surrounded by a clear rim of about 200 Å below the vesicular membrane There are only few granulated vesicles per ending. The clear vesicles are homo-

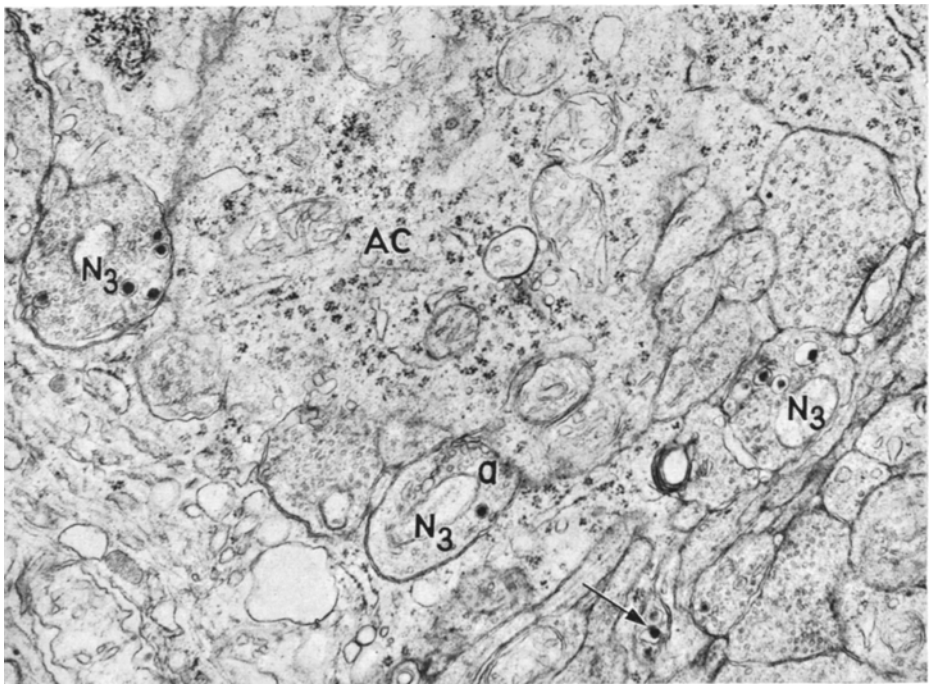

Fig. 4. Amacrine cell surrounded by several type 3 nerve endings, some of which contain large granulated vesicles. An active point is seen in a contact between a type 3 ending and the amacrine cell cytoplasm. In the adjacent inner plexiform layer thin axons are observed, one of them contains large granulated vesicles (arrow). × 26000

genously distributed or, more frequently, they are concentrated at special sites near the presynaptic membrane adjacent to type 1 endings (Fig.2) or to ganglion cell dendrites (Fig. 3). Type 2 endings are less frequently observed on ganglion cells soma, but in this case, no "active sites" with vesicular concentration were observed. In primates DOWLING and BOYCOTT (1965) had observed endings with the same morphology, but without describing the presence of granulated vesicles. They interpreted them as belonging to amacrine cells.

c) Type 3 ending is concentrated in the outermost portion of the inner plexiform layer (Fig. 4). These terminals range between 1 to 2.5 μ and have a mixed population of vesicles, with clear ones of about 460 Å and granulated vesicles of about 1150 Å (range between 750—2300 Å). There is an average of 4—5 granulated vesicles per terminal (Fig. 5). However, the relative proportion of clear and granulated vesicles is variable in this type of ending, ranging from a predominance of granulated vesicles to a complete absence of them. This finding may be due to the incidence of the plane of section because the other characteristics of the terminal are similar to those of type 3 endings.

Type 3 endings are usually observed in contact with amacrine cells at the edge of the inner plexiform layer (Fig. 4). Few similar axons and terminals have been found in deeper regions of the inner plexiform layer (Fig. 6), within the inner nuclear layer, near the outer plexiform, and between ganglion cells and the layer

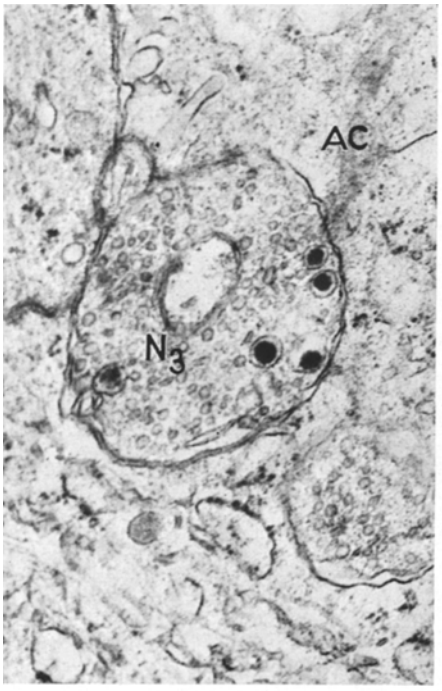

Fig. 5

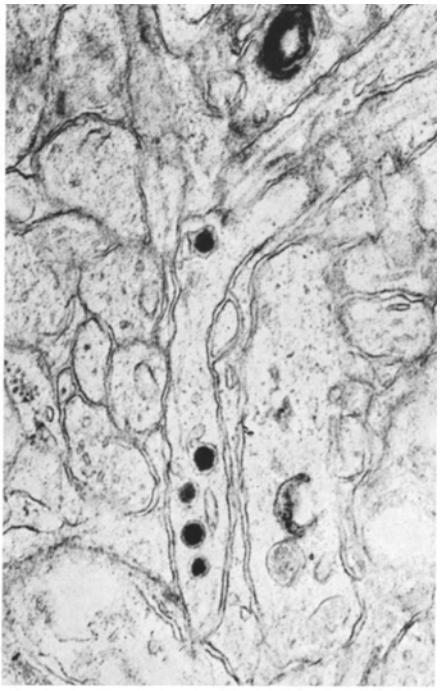

Fig. 6

Fig. 5. Higher magnification of a small area of Fig. 6 showing a type 3 nerve ending with clear and some large granulated vesicles in close contact with amacrine cell cytoplasm. × 72000

Fig. 6. A thin axon within the inner plexiform layer containing large granulated vesicles, similar to those observed in type 3 endings. × 42000

of optic fibers. As it will be discussed later, these granulated fibers and terminals — which have not been described before — are interpreted as corresponding to the centrifugal fibers of central origin.

Granulated vesicles in perikarya of the inner nuclear layer

Some of the cells present in the amacrine cell layer of Cajal contain granulated vesicles in the cytoplasm. They are characterized by an irregular nucleus and well developed Golgi complex which is in the region of the cytoplasm directed toward the inner plexiform. The granulated vesicles, with a mean diameter of 1050 Å (range 700—1500 Å), are distributed throughout the perikaryon but more frequently in the vicinity of the Golgi complex (Fig. 7). Lysosome-like bodies of 1200—4000 Å with a granular matrix surrounded by a single membrane are observed in the same region.

Some cells with granulated vesicles are found adjacent to the inner plexiform layer with a characteristic "bottle" shape. Here the granulated vesicles are less abundant and limited to Golgi zone. Primary branches emerging from the perikarya of these cells have been observed and are characterized by the presence of neurotubules, ribosomes, mitochondria, lysosomes and granulated vesicles of 766 Å.

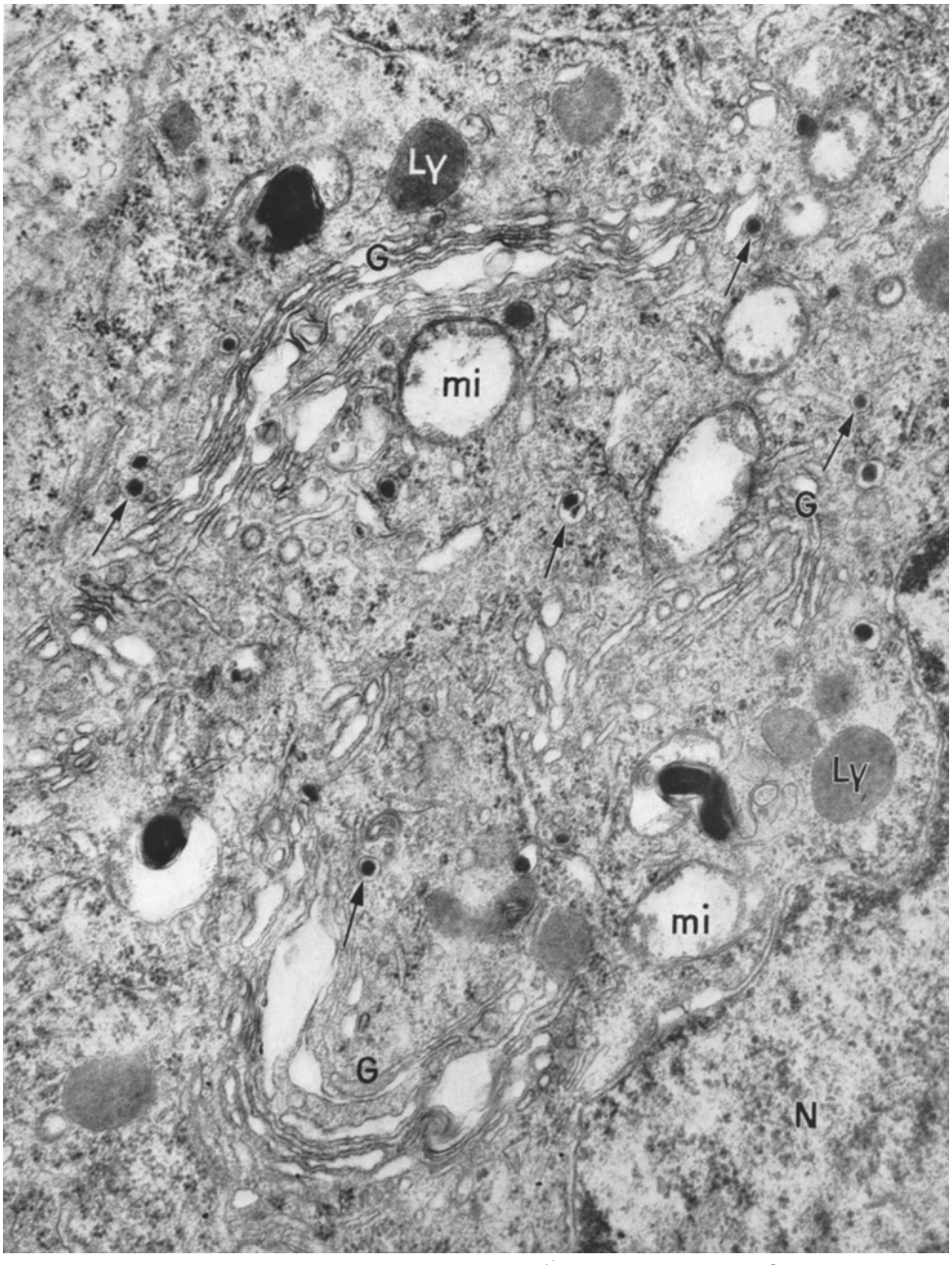

Fig. 7. Golgi region of one of the cell types found in the amacrine layer. In close association with Golgi cisternae, there are granulated vesicles (arrows), lysosomes of different size and density, and some mitochondria. × 45000

Granulated vesicles of about 900 Å are also found in some large cell processes penetrating into the inner plexiform layer (Fig. 8). The perikaryon of these cells has a bipolar shape and usually does not contain lysosomes or granulated vesicles.

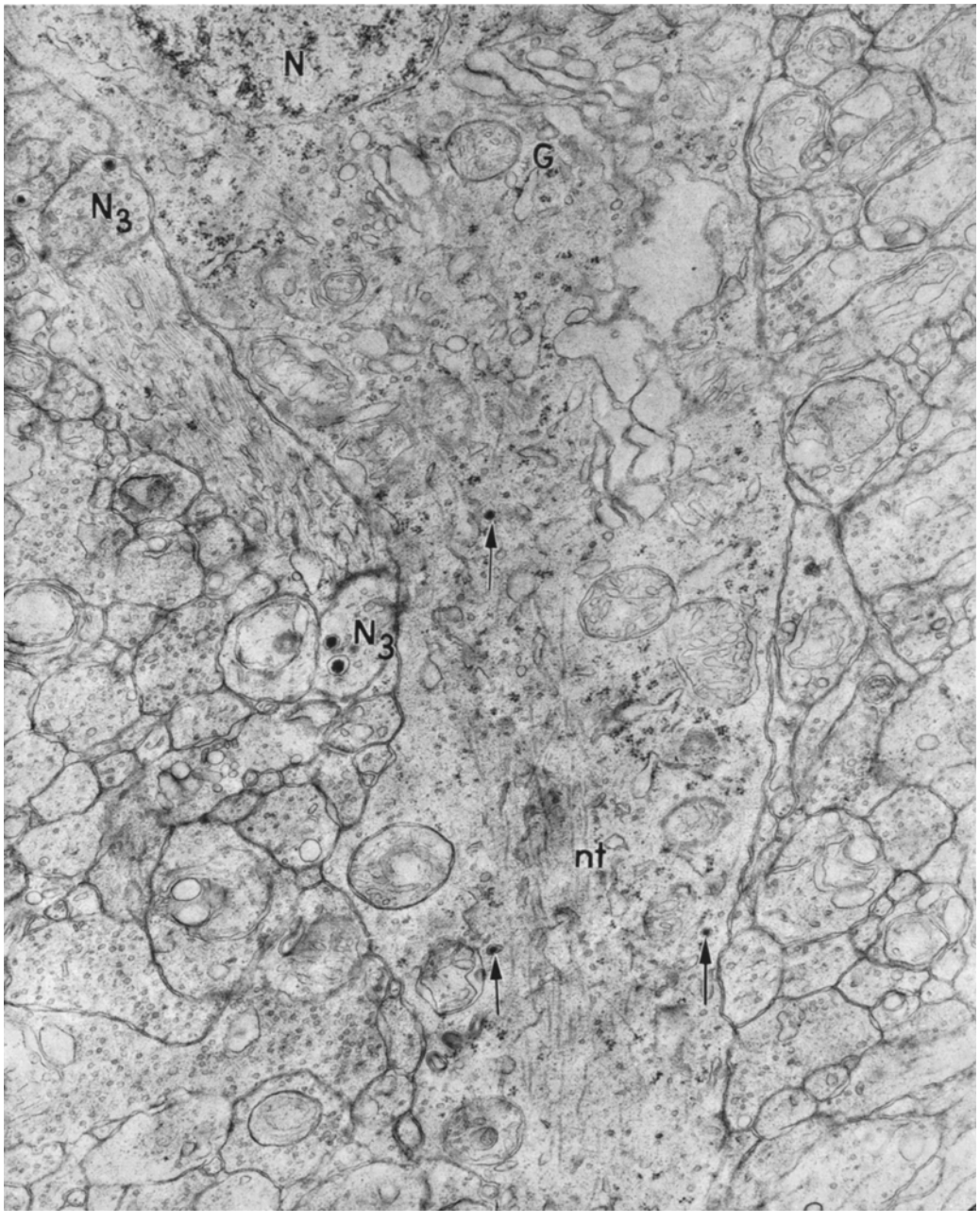

Fig. 8. Descending main process from a bipolar shaped cell found in the amacrine layer. Large Golgi complex, several mitochondria, neurotubules and few small granulated vesicles (arrows) are observed. Two type 3 nerve endings are in contact with this cell. × 30000

Granulated vesicles in perikarya of the ganglion cell layer

Under the electron microscope, two clearly defined types of neurons are observed in the ganglion cell layer. One is characterized by an irregular contour, a

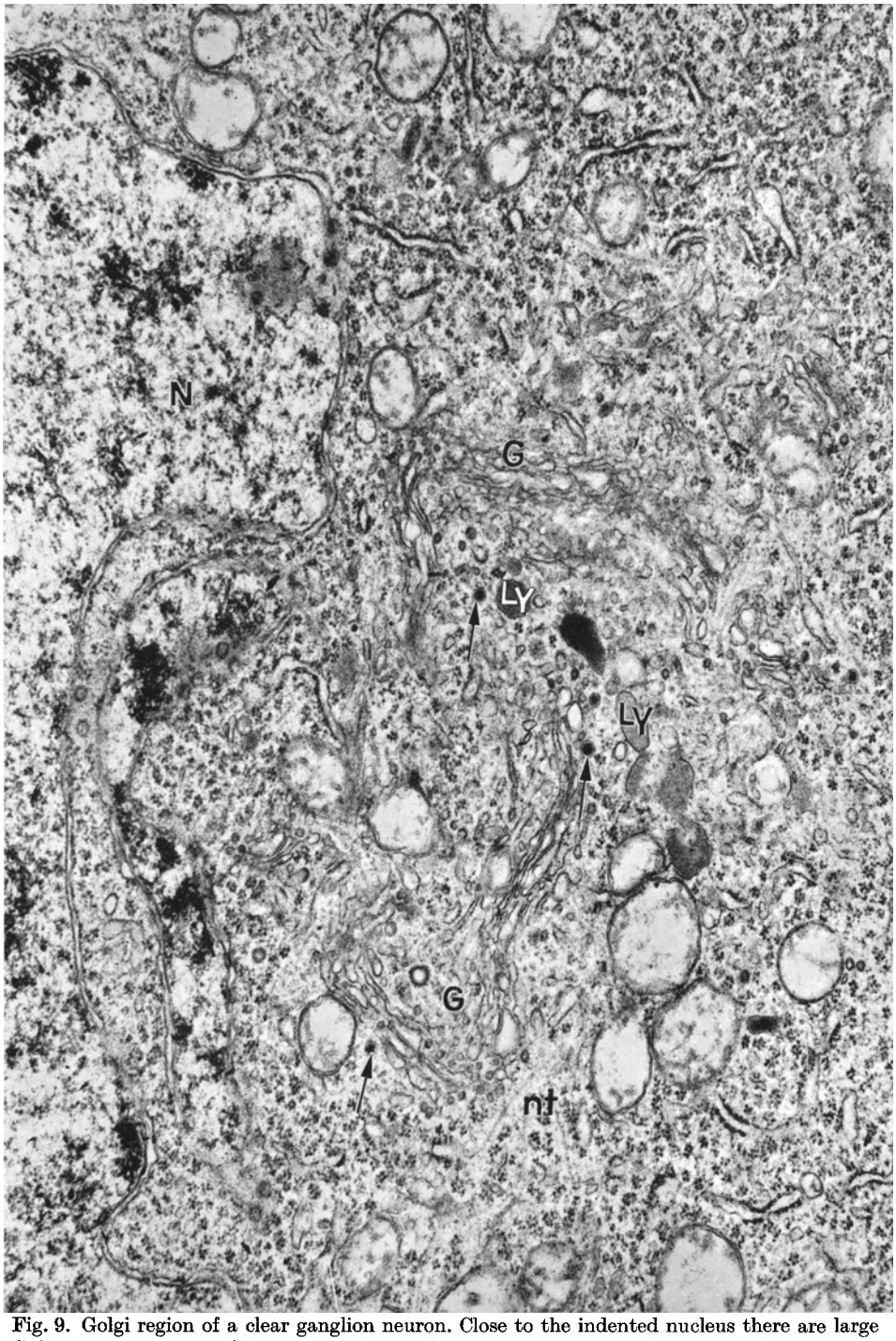

Fig. 9. Golgi region of a clear ganglion neuron. Close to the indented nucleus there are large Golgi cisternae surrounded by many clear and some granulated vesicles (arrows), lysosomes of different sizes and densities, mitochondria, numerous neurotubules and ribosomes may be seen. × 42500

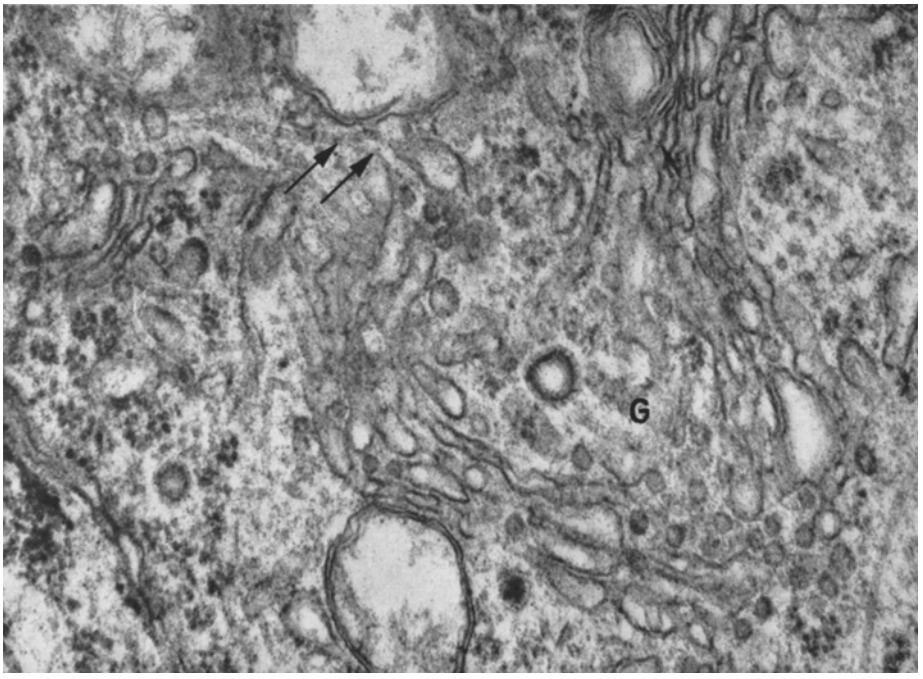

Fig. 10. Detail of Fig. 9 at a higher magnification, showing the close relationship of neurotubules with the Golgi cisternae. The double arrow indicates a neurotubule ending in a large Golgi vesicle. ×52500

dense cytoplasmic matrix extremely rich in ribosomes, numerous mitochondria and dilated sacs of the endoplasmic reticulum. In contact with such ganglion cells large type 1 terminals are observed.

The second type of neuron is spherical, has a clear cytoplasmic matrix with less number of ribosomes and mitochondria and a prominent nucleolus. Such neurons have large Golgi zones containing, in addition to flattened cisternae, numerous clear and some granulated vesicles of 600—800 Å (Fig. 9). In the same region there is a concentration of lysosomes and neurotubules which converge toward the Golgi complex (Fig. 10). The close relationship between neurotubules and Golgi complex has been previously suggested by MORI (1966). On these cells large type 1 endings, coming from rod-bipolars, may be observed. Sometimes within the optic fiber layer it is possible to observe granulated vesicles of 800 Å.

Discussion

The evidence presented here demonstrates that there are three different types of nerve endings in the inner plexiform layer of the rat retina, two of which, i.e. types 2 and 3, contain granulated vesicles. While both contain a mixed population of vesicles, these two types of endings may be distinguished by the diameter of the granulated vesicles (640 and 1140 Å respectively in types 2 and 3), their localization within the layer and by their different connections. Type 2 terminals are connected with bipolar cell endings, dendrites and perikarya of ganglion cells and

may form complex glomerular-like synapses. Type 3 endings contact with processes and somata of amacrine cells of Cajal.

The granulated vesicles present in these nerve endings are larger than those first found in adrenergic peripheral endings by DE ROBERTIS and PELLEGRINO DE IRALDI (1961) and more similar in size to those described in the anterior hypothalamus (PELLEGRINO DE IRALDI et al., 1963) and in different autonomic territories (HAGER and TAFURI, 1959; TAXI, 1961; GRILLO and PALAY, 1962). Such granulated vesicles correspond to the *intermediate type* of PELLEGRINO DE IRALDI and DE ROBERTIS (1964); denomination used to differentiate them from the small adrenergic vesicles and the large granulated droplets of the adrenal medulla.

It is of great interest to correlate these observations with those of MALMFORS (1963) in rats, HÄGGENDAL and MALMFORS (1965) in rabbits and EHINGER (1966 a, b) in monkeys, rats, rabbits and guinea pigs who used the histochemical fluorescence technique for catecholamines of Falck and Hillarp. There is a remarkable correspondence in the localization of the granulated vesicles and the specific fluorescence in the outermost zone of the inner plexiform layer, where type 3 nerve endings are located, and in some neurons of the inner nuclear and ganglion cell layers. This relationship is less evident in the other sites where granulated vesicles are found, a fact that may be possibly explained by a low concentration of catecholamines, below the sensitivity of the fluorescence method. The histochemical technique does not separate fluorescence due to dopamine from that of noradrenaline, but the findings of HÄGGENDAL and MALMFORS (1963, 1965) tend to support the view that dopamine is the predominant catecholamine in the retina of rabbits, and PELLEGRINO DE IRALDI and ZIEHER (1966) found a high content of dopamine in the retina of rats.

Several pieces of evidence favour the view that the granulated vesicles of intermediate size may contain catecholamines. DE ROBERTIS et al. (1965) found a large number of such vesicles in the vesicular fraction isolated from the anterior hypothalamus, which also has the highest concentration of norepinephrine. By radioautography at the electron microscope level, AGHAJANIAN and BLOOM (1966) localized exogenous tritiated norepinephrine in the vicinity of granulated vesicles in hypothalamus nerve endings. Reduction in number of granulated vesicles of the hypothalamus, with a norepinephrine releaser, was observed by MATSUOKA et al. (1965); while an increase in the number of such vesicles, after DOPA and MAO inhibitors, was observed by CLEMENTI et al. (1966) in sympathetic ganglia and by BAK and HASSLER (1966) in substantia nigra and caudate nucleus of mice. This last finding has been confirmed by us in the rat. Although the correspondence between granulated vesicles and catecholamines is still questioned in some regions of the CNS (LENN, 1965; FUXE et al., 1966), in the retina, the coincidence between granulated vesicles and localization of specific fluorescence is highly suggestive and indicates that these vesicles may represent the site of storage of catecholamines.

The coexistence of two types of vesicles — clear and granulated — within the same nerve ending has been observed, among other locations, in different autonomic territories (TAXI, 1961; GRILLO and PAALY, 1962), in the synaptic areas of invertebrates (PELLEGRINO DE IRALDI and DE ROBERTIS, 1962; GERSCHENFELD, 1962), in the anterior hypothalamus (PELLEGRINO DE IRALDI et al., 1963) and in

the median eminence of rats (KOBAYASHI et al., 1966; RINNE, 1966). PELLEGRINO DE IRALDI and DE ROBERTIS (1962) interpreted this coincidence as suggestive of the presence of more than one transmitter in a single terminal. Such a hypothesis has recently received some experimental support from CLEMENTI et al. (1966), who observed an increase in granulated vesicles in the cholinergic presynaptic terminals of sympathetic ganglia after treatment with DOPA and iproniazid.

Because of their localization, type 3 nerve endings could originate either from the associational amacrine cells of Cajal or from centrigufal fibers. Although the first possibility is favored by the presence of similar granulated vesicles in some cells of the amacrine cell layer, associational amacrine cells have not been observed in the retina of mammals. Moreover the number of cells that contain granulated vesicles is relatively small. According to the description of CAJAL (1904), the centrifugal fibers end in contact with cells of the inner nuclear layer in a way similar to that observed by us for nerve endings of type 3. This is the only synaptic connection revealed by the electron microscope in contact with amacrine cells. The nerve fibers with granulated vesicles found in the deeper zones of the inner plexiform layer may represent ascending centrifugal fibers. MALMFORS (1963) observed the persistence of fluorescence after opticotomy and superior cervical ganglionectomy and interpreted that the terminals were intraretinal in origin. This experiment should not be considered as definitive because the period of time elapsed between the opticotomy and the observation was very short (48 hours) and does not guarantee the degeneration of all nerve endings as demonstrated by DOWLING and COWAN (1966). Recently in the retina of the pigeon, we have observed (PELLEGRINO DE IRALDI and JAIM ETCHEVERRY, 1967 b) granulated vesicles localized in nerve endings which correspond to those identified by DOWLING and COWAN (1966) as belonging to centrifugal fibers of central origin.

The type 2 nerve endings that contain smaller granulated vesicles, are similar to the amacrine cell terminals of primates (DOWLING and BOYCOTT, 1965). The lack of finding of granulated vesicles reported by these authors may be due to the different species, and more probably to the fixation technique. Another possibility is a physiological variation due to a circadian rhythm. Preliminary observations suggest that there are significant differences in the distribution of granulated vesicles during day and night.

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