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Ultrastructural Changes during Early Development of Retinal Ganglion Cells in *Xenopus**

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Summary. All cells in the optic vesicle of *Xenopus* embryos from stages 27 to 31 have the same ultrastructure. They are elongated and appear to extend from the internal to the external surfaces of the optic vesicle. They are bound together by terminal bars at the internal (lumen) margin, have microvilli and a cilium on the internal margin, and are covered with a basement membrane on the external margin. Their cytoplasm contains abundant free ribosomes, polysomes, mitochondria, yolk and lipid inclusions, and sparse endoplasmic reticulum.

Although other studies have shown that retinal ganglion cells originate at stages 29—30 and have their central connections determined before stage 31, these events could not be correlated with any ultrastructural changes. The first sign of differentiation in retinal cells was an increase in endoplasmic reticulum and Golgi apparatus at stage 32. Microtubules and microfilaments appeared at stage 33 in association with the first axonal outgrowth from retinal ganglion cells. Cytodifferentiation proceeded gradually until large areas of Nissl substance had developed by stage 35. At larval stage 48 the ganglion cells resembled those in the adult.

Key-Words: Development — Neurons — Retina — *Xenopus*.

Introduction

The purpose of this investigation was to examine the ultrastructure of neuroepithelial germinal cells in the optic vesicle and developing retina of *Xenopus laevis*, and to study the changes that occur during differentiation of the retinal ganglion cells.

Autoradiographic studies have shown that 100% of the cells of the optic vesicle of *Xenopus* embryos before stage 29 incorporate thymidine into DNA (Jacobson, 1968a). This shows that the optic vesicle is composed entirely of germinal cells, and in this investigation it has been assumed that all cells of the developing retina at stages 27 and 28 were germinal cells.

In *Xenopus* the production of ganglion cells commences at embryonic stage 29, for at that time some cells are produced which have ceased DNA synthesis and mitosis, and which will migrate to take up the positions of the adult retinal ganglion cells. These cells eventually differentiate into adult retinal ganglion

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cells (Jacobson, 1968a). Since these cells may be distinguished from the germinal cells on the basis of their position, a comparison could be made between the ultrastructure of the germinal cells and the newly formed ganglion cells. One of the aims of this investigation was to establish whether there are any ultrastructural differences between the germinal cells and the young retinal ganglion cells, and to determine when such differences arise.

As the ganglion cells are produced, or shortly afterwards, their central connections with the optic tectum become specified. This specification process occurs at stages 30 and 31 (Jacobson, 1967, 1968b) before any ganglion cell differentiation is detectable with light microscopy and before the outgrowth of optic axons. The question remains whether specification is accompanied by structural changes which might be detected with electron microscopy. A careful examination of the germinal cells and young ganglion cells was thus made during the time of ganglion cell specification. In addition, the ganglion cells were examined at various later stages of their differentiation, up to and including the adult stage. This represents a systematic attempt to investigate structural changes in a portion of the developing nervous system where the precise time of important developmental events (ganglion cell production and specification) is known.

Materials and Methods

Embryos of *Xenopus laevis* were selected by stage according to the normal tables of Nieuwkoop and Faber (1956). Embryos of stages 27, 28, 29, 30, 31, 32, 33, 34, 35, 48, and four adult retinas were prepared for electron microscopy. Sections were cut from the eyes of 70 embryos and 4 adult animals. The whole embryo or the excised retinas of adults were fixed by immersion in 2% glutaraldehyde buffered with veronal acetate. Fixation was for 1 hour at 0—4° C. The eyes were dissected from the embryos or the adult retina was dissected into small pieces and postfixed in 1% osmic acid buffered with veronal acetate. The tissue was dehydrated in a graded series of acetone and imbedded in Vestopal-W. During dehydration the tissue was stained for 1 hour in 70% acetone saturated with uranyl acetate. Thin sections were cut with a Porter-Blum-MT-2 microtome, collected on grids and stained with either lead citrate (Millonig, 1961) or lead tartrate (Reynolds, 1963) for 5—10 minutes. For purposes of finding the desired position in the embryonic eye 1 micron thick sections were cut and examined by light microscopy until the central portion of the optic vesicle was reached. Thin sections were only collected from the central portion of the embryonic retinas. Specimens were examined in a modified RCA-EMU 3C electron microscope.

Results

Stages of Germinal Cell Proliferation

Before stage 29 the optic vesicle is composed solely of proliferating retinal germinal cells (Figs. 1—3). During stages 27 and 28 the germinal cells have a characteristic elongated shape with ovoid nuclei (Fig. 1). The ends of the cells which border on the lumen of the optic vesicle are irregular and often have microvilli or a cilium which projects into the lumen (Fig. 1). The cells are joined by terminal bars at the lumen border (Fig. 3). The outer border of the optic vesicle which eventually becomes the vitreal border of the retina will be referred to as the vitreal border. The ends of the germinal cells which form the vitreal border are smooth, with no projections, and are covered by a basement membrane. The cells are not joined by terminal bars on this border. The cytoplasmic

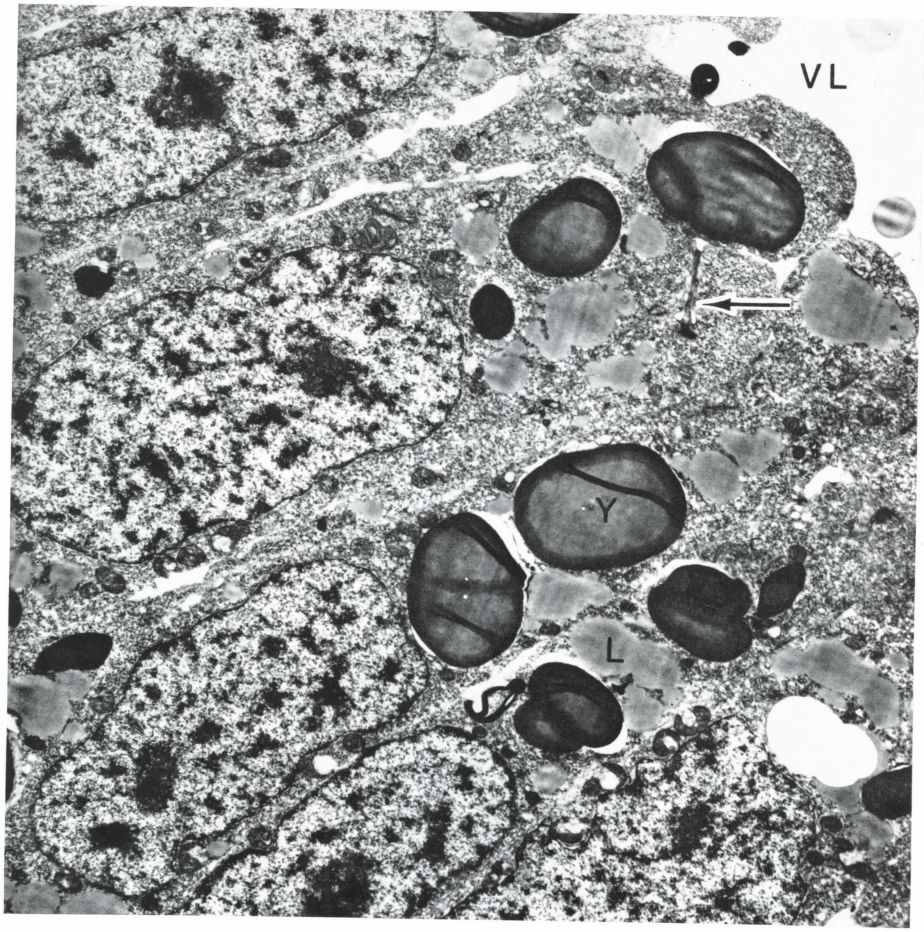

Fig. 1. Optic vesicle of *Xenopus* embryo at stage 27 showing germinal cells. Cilium (arrow) lipid droplet (*L*), optic vesicle lumen margin (*VL*), yolk inclusion (*Y*). 5,000×

structure of the germinal cells is characterized by many free ribosomes, mitochondria, and electron-dense inclusions of yolk and lipids (Figs. 1, 2). The germinal cells contain few cytoplasmic membranes, but small amounts of smooth and rough endoplasmic reticulum can be found in most cells (Fig. 2). The other type of cytoplasmic membranes found were very small Golgi zones located near the lumen border of the germinal cell layer. Only two such zones were encountered during this investigation. No microtubules or microfilaments were found in the germinal cells except for filaments projecting from terminal bars (Fig. 3).

Stages of Ganglion Cell Production and Specification

Cells of the optic vesicle during stages 29 to 31 appeared structurally similar to cells at earlier stages (Figs. 4—7). It was not possible to detect definite signs of differentiation although it is known from other studies (Jacobson, 1968a, b)

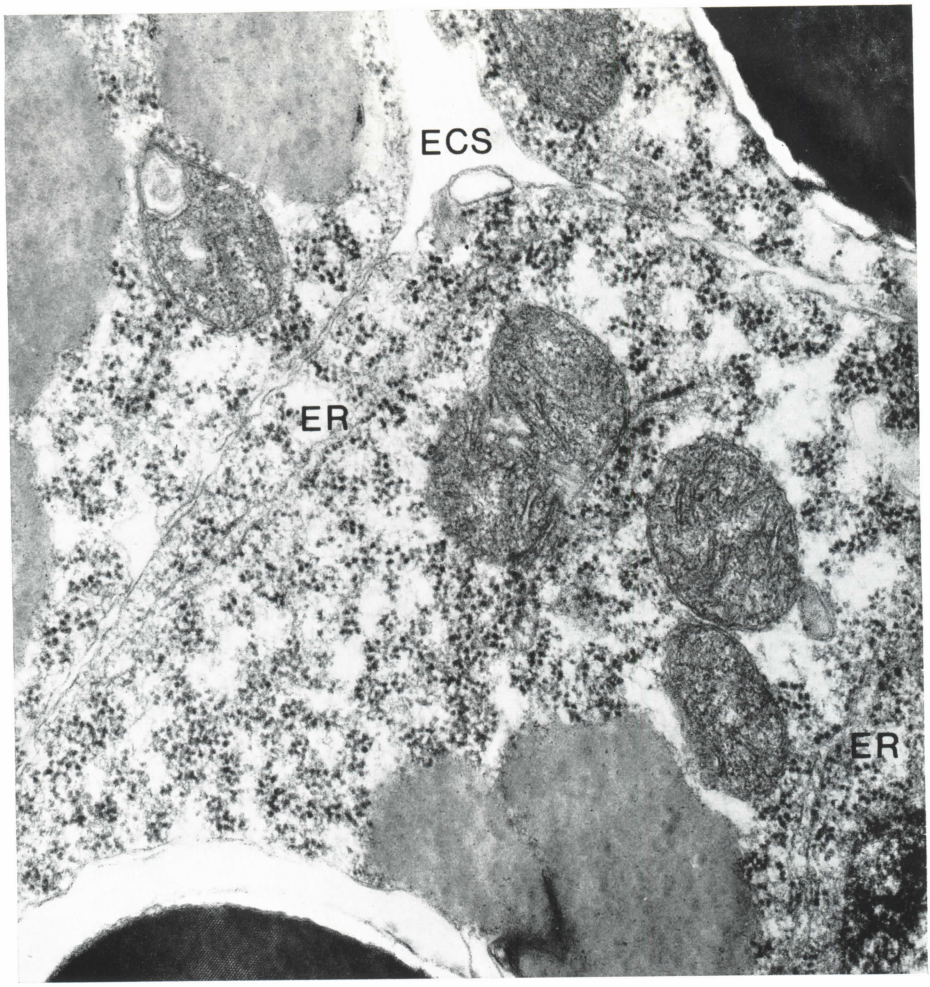

Fig. 2. Germinal cell of optic vesicle at stage 28, showing sparse endoplasmic reticulum (*ER*), and large extracellular space (*ECS*) where three cells meet. The yolk inclusion at lower left has a crystalline array. 22,000×

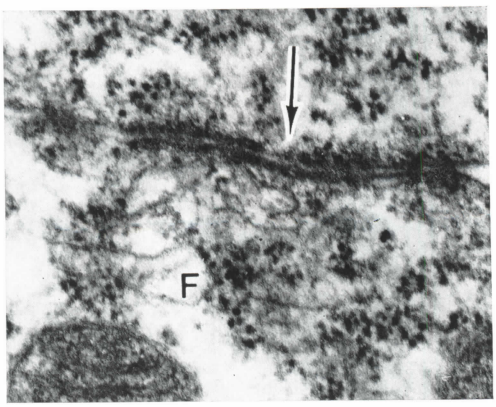

Fig. 3. Terminal bar (arrow) joining two germinal cells in optic vesicle of a stage 28 embryo. Microfilaments (*F*) radiate from the terminal bar. 32,000×

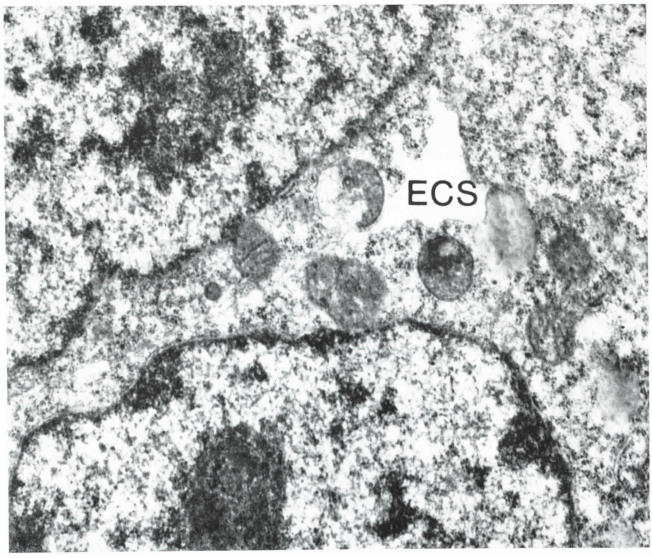

Fig. 4. Germinal cells of optic vesicle of a stage 29 embryo showing abundant free ribosomes, lack of cytoplasmic membranes, and wide extracellular clefts (*ECS*) where three cells meet. 9,000×

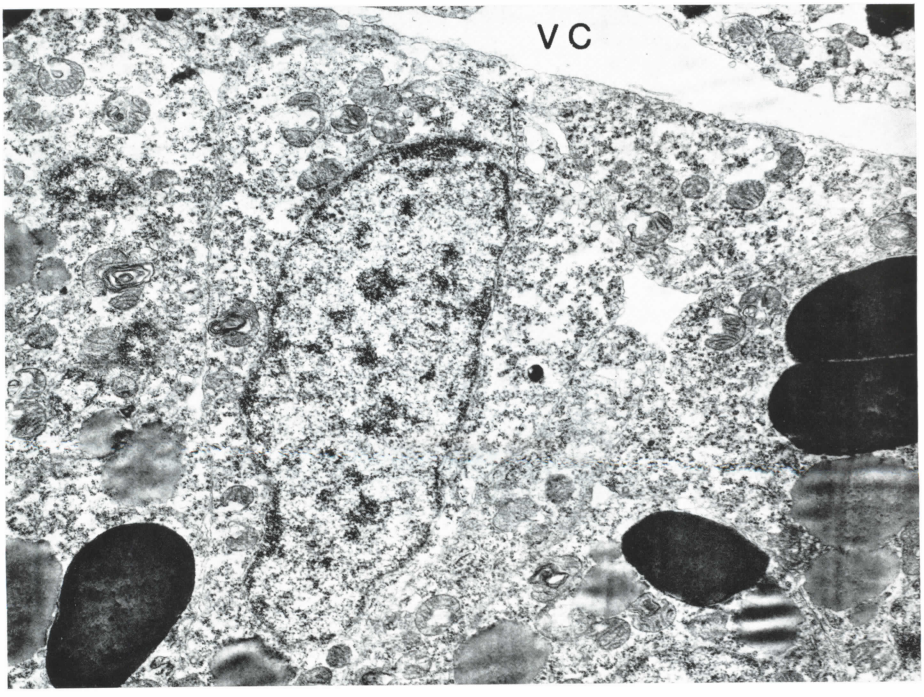

Fig. 5. Cells in mantle layer of optic cup of stage 30 embryo. The cells have ultrastructural features similar to those at earlier stages. Vitreal cavity (*VC*). 7,000×

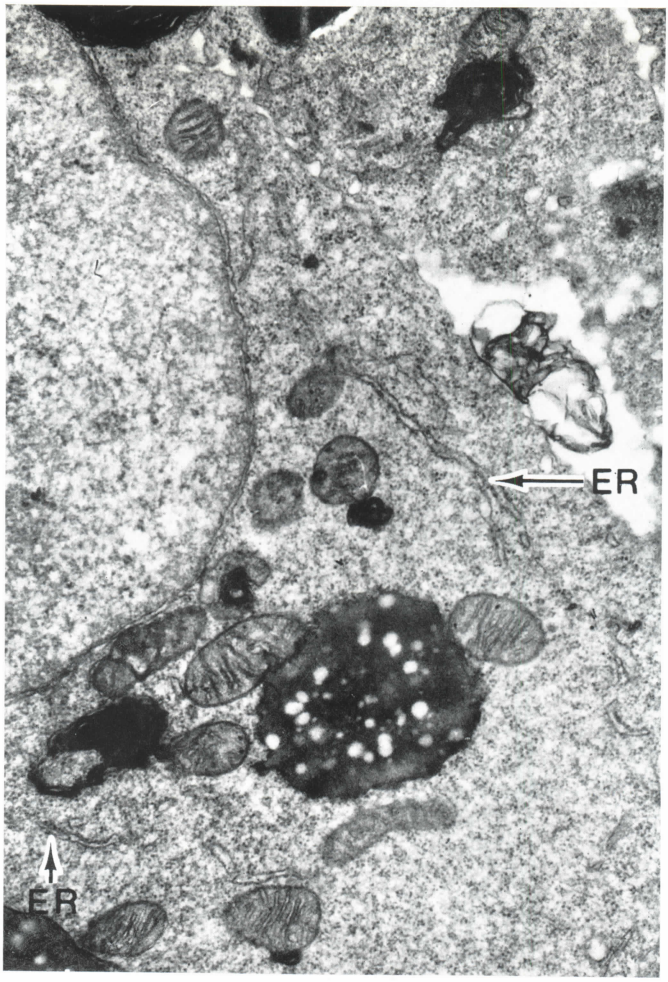

Fig. 6. Cell in mantle layer of optic cup of stage 30 embryo. The cell has about the same amount of endoplasmic reticulum (*ER*) as the cell at stage 28 in Fig. 2. 20,000×

that ganglion cells were being produced at stages 29 and 30 and that their connections in the tectum were predetermined at stages 30 and 31. At all stages examined some cells had relatively larger amounts of endoplasmic reticulum than others, but a comparison of Fig. 2 (stage 28 embryo) with Figs. 6 and 7 (stages 30 and 31 embryos) shows that there was no way of determining the stage of the embryo at stages 28 through 31 from the ultrastructure of the retinal cells.

Stages of Ganglion Cell Maturation

Beginning with stage 32 the cells forming the mantle layer of the optic vesicle showed definite signs of differentiation into ganglion cells. There was an obvious increase in the amount of cytoplasmic membranes in the form of both endoplasmic reticulum and Golgi zones (Fig. 8). There was a particularly dramatic

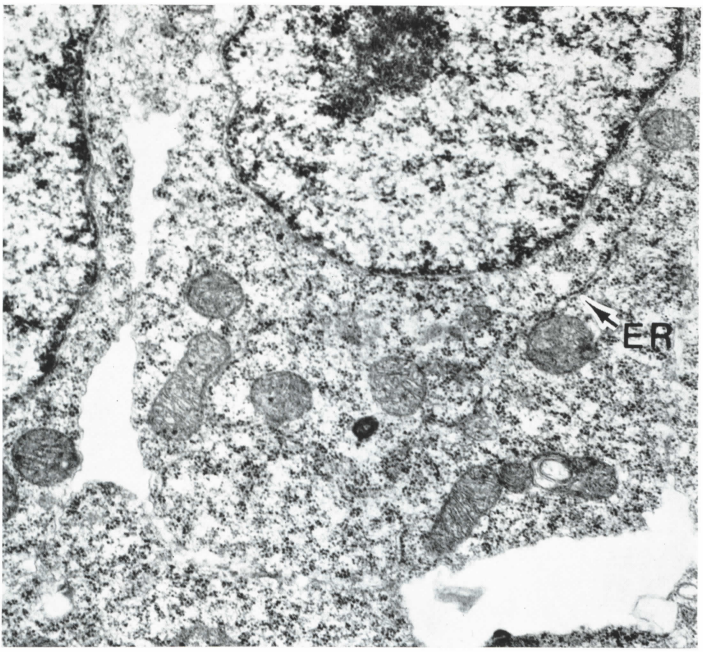

Fig. 7. Cells in mantle layer of optic cup of stage 31 embryo. Note sparse endoplasmic reticulum (*ER*), abundant free ribosomes, and large extracellular space where three cells meet. 14,000×

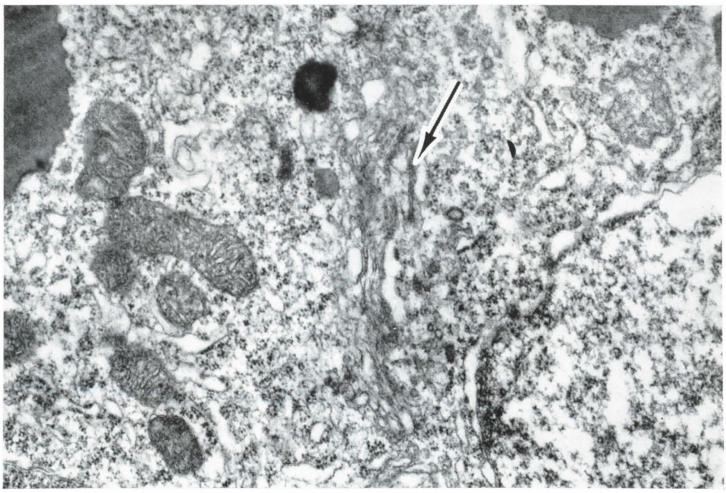

Fig. 8. Golgi apparatus (arrow) in a retinal ganglion cell of stage 32 embryo. 17,000×

increase in the size and extent of the Golgi zones when compared with these structures in the germinal cells. This trend continued after stage 32, and the proliferating endoplasmic reticulum began to constitute large areas of Nissl substance by stage 35 (Fig. 9).

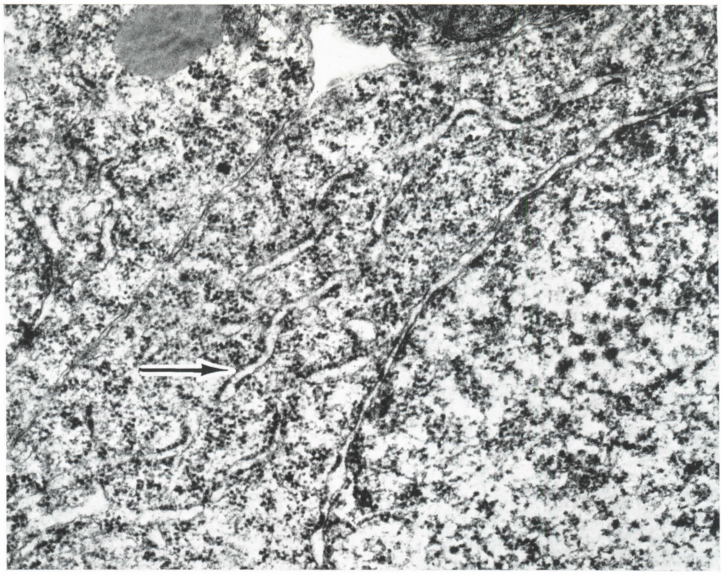

Fig. 9. Abundant rough endoplasmic reticulum (arrow) in retinal ganglion cell of stage 33 embryo. 20,000×

Cell outgrowths which could be identified as optic axons first appeared at stage 33 (Fig. 10). These outgrowths contained microtubules of about 250 Å diameter and microfilaments of about 80—90 Å diameter.

In retinas of stage 48 embryos an apparent decrease in the number of cytoplasmic membranes and organelles had occurred (Fig. 11). Up to stage 35 there were increasingly large areas of rough endoplasmic reticulum and a profusion of free ribosomes, but these were much sparser at stage 48. The Golgi apparatus was the most prominent organelle seen at stage 48 (Fig. 11), though each cell still contained some free ribosomes, mitochondria, and endoplasmic reticulum. Microtubules often extended from the perikaryon into axons and dendrites.

Ganglion cells in the adult retina contained few profiles of endoplasmic reticulum, few free ribosomes, but extensive Golgi zones. The latter were often located near the point of exit of a ganglion cell process. Microtubules commonly extended from the cell body into the processes. Some ganglion cells had a cilium projecting into the inner plexiform layer.

Discussion

The first structural signs of differentiation may be seen in the retinal ganglion cells in *Xenopus* embryos at stage 32. This is about 5 hours after the beginning of ganglion cell production and $3^1/_2$ hours after the completion of ganglion cell specification. Thus, newly produced ganglion cells must exist in the embryonic retina for several hours before morphological differentiation is detectable. However, during these hours the cells are undergoing changes of an unknown nature which result in a precise specification of their projection onto the optic tectum. It must be concluded that the newly formed ganglion cells cannot be distinguished from their predecessor germinal cells on the basis of morphology

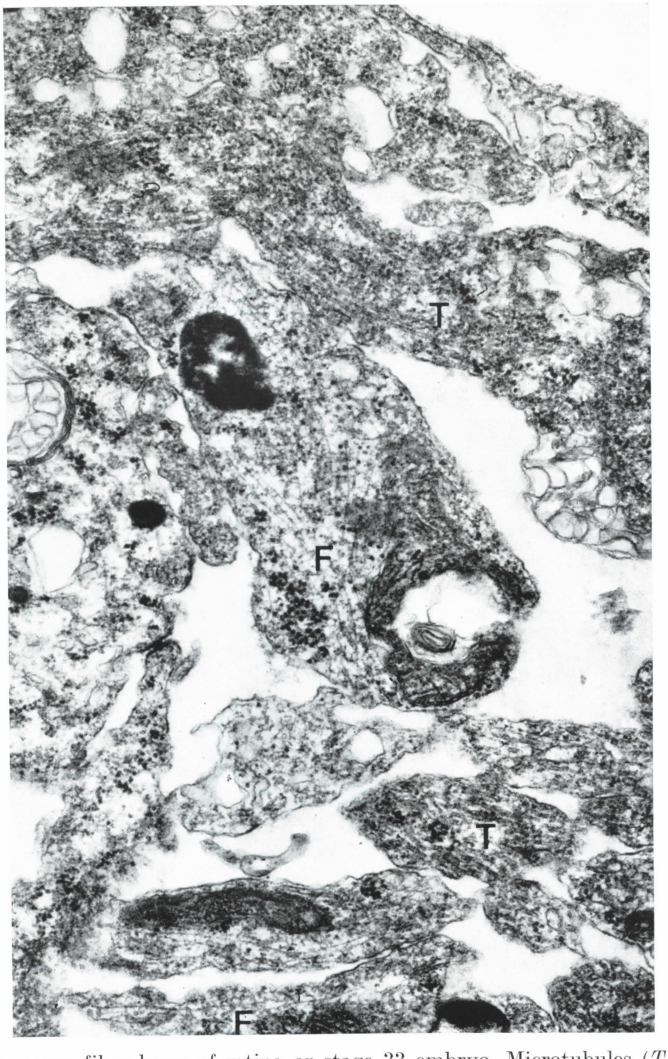

Fig. 10. Optic nerve fiber layer of retina or stage 33 embryo. Microtubules (T) and microfilaments (F) are shown in the growing optic axons. 25,000×

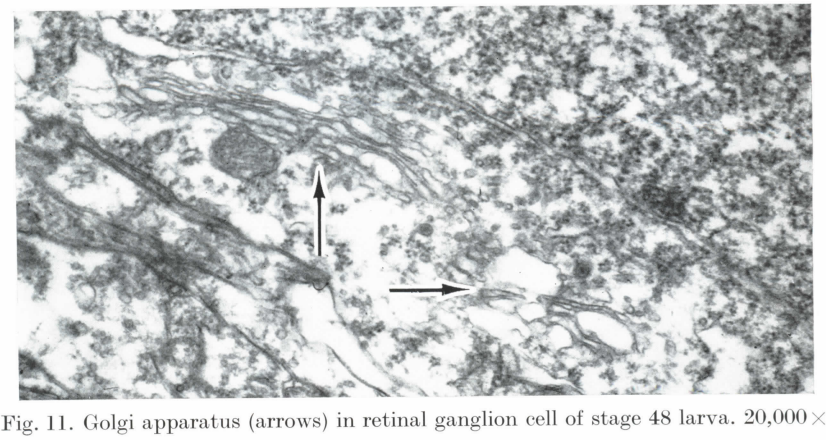

Fig. 11. Golgi apparatus (arrows) in retinal ganglion cell of stage 48 larva. 20,000×

alone. This is in agreement with the conclusions reached by Wechsler (1967) from observations on embryonic chick brain. This means that the first identifiable sign of neuronal differentiation in retinal ganglion cells is specification of connections and not a morphological change. It is not known if all neurons undergo this specification process or not. Although the specification process may be accompanied by, or be reflected in, some biochemical changes, these changes could not be resolved by the methods employed in this investigation. The results of this study indicate that a cell in the process of being specified appears identical to all other cells in the population.

In *Xenopus* embryos before stage 32 all cells of the optic vesicle have ultrastructural characteristics of immature cells, namely an elongated shape, high nuclear-cytoplasmic ratio, large amounts of free ribosomes and polysomes, and general paucity of cytoplasmic membranes. Porter (1961) considered the preponderance of free ribosomes and lack of endoplasmic reticulum as useful means of identifying proliferating, undifferentiated cells. It is impossible to decide at this time whether the germinal cells prior to stage 29 form a structurally homogeneous population as Fujita (1963, 1964) and Wechsler (1967) have concluded.

A Golgi zone was only detected in two germinal cells of the many that were examined. It seems unlikely that this structure is present continuously in all germinal cells or it would have been seen more often, especially since particular attention was given to it during this investigation. There were great differences in the amount of endoplasmic reticulum in different germinal cells. Although this apparent heterogeneity may be due to the sampling errors inherent in any electron microscopical study, it does raise enough doubt to make any conclusions concerning the homogeneity of the retinal germinal cells seem premature. This poses the question of the genetic homogeneity of the germinal cells. Are all germinal cells equally capable of forming any component of the retina or do some germinal cells have a restricted capacity to produce only one type of retinal cell? This problem cannot be resolved by morphological studies alone, and is one of the fundamental problems of neuroembryology.

The first structural sign of differentiation in the young ganglion cells was the increase in cytoplasmic membranes, an observation that is in agreement with results reported by others (Belairs, 1959; Tennyson, 1962, 1965; Eschner and Glees, 1963; Meller *et al.*, 1965, 1966, 1967; Fujita, 1965, 1966a, 1966b; Wechsler, 1965, 1966). However, the increase in endoplasmic reticulum and Golgi apparatus was not detectable until stage 32 or about 5 hours after the ganglion cells were produced at stages 29—30. There may have been a slow accumulation of endoplasmic reticulum during stages 30 and 31 but this was not sufficient to allow conclusive identification of the young ganglion cells.

The next phase of ganglion cell differentiation, i.e., the growth of optic axons, coincided with the time of first appearance of microtubules and microfilaments in the young ganglion cells. This sequence of events would suggest that the increased endoplasmic reticulum, the appearance of the large Golgi zone, and the first appearance of microtubules and microfilaments may be functionally correlated with the growth of the optic axons. In the adult retinal ganglion cells a large Golgi zone was almost always found near the point of exit of a cell process. Ramón y Cajal (1929) commented on the presence of the Golgi apparatus at the

site of axon outgrowth. Tennyson (1962) reported that the Golgi zone was often found near the point of axon outgrowth in the rabbit embryo. The significance of the Golgi apparatus in transferring proteins from their site of synthesis in the ribosomes of the Nissl substance to the axon has been reviewed by Droz (1969).

Microtubules and filaments have been implicated in many functions in other types of cells, including cell motility (Freed *et al.*, 1968; Behnke, 1965), cell support (Slautterback, 1963), cytoplasmic flow or streaming (Slautterback, 1963; Taylor, 1965; Nagai and Rebhun, 1966), and pseudopod extension and contraction (Wohlman and Allen, 1968). All of these functions could be important to a cell in the process of growing long axons. Other investigators have reported the presence of microtubules and/or microfilaments in germinal cells (Herman and Kauffman, 1966; Wechsler, 1967; Lyser, 1968). Sechrist (1969) considers the presence of microfilaments to be an early sign of differentiation of the young neuron. Neither microtubules nor filaments were found in the cells of the *Xenopus* retina prior to stage 33 except for the fine filaments around the terminal bars. The other authors reported results on chick and mouse embryos and the differences between their results and ours may reflect differences among the species investigated.

The results of this study also seem to indicate that the extracellular space decreased as the retina matured. The intercellular clefts were very wide in the embryonic tissue, particularly where several cells come together (Figs. 2, 4), but in the adult the intercellular cleft had the usual dimensions seen after glutaraldehyde fixation of adult nervous tissue. Others have reported that the 200 Å clefts typical of adult central nervous system are present in the chick embryo nervous system (Bellairs, 1959; Wechsler, 1966; Mugnaini and Forströnen, 1967). However, our results are more in agreement with those of Karlsson (1967) and del Cerro *et al.* (1968a, 1968b) who reported a reduction in the width of the intercellular clefts during development of the rat lateral geniculate nucleus and cerebellum.

The structure of adult retinal ganglion cells described in this report agrees with that reported by Yamada (1957) and Dowling (1968) for the retinal ganglion cells of other amphibians. The decrease in cytoplasmic organelles observed after stage 48 would seem to reflect the changing requirements of the ganglion cells. According to Gaze and Peters (1961) the optic axons of *Xenopus* probably form their connections in the optic tectum at stage 49. This means that by stage 48 the ganglion cells are nearing the end of the period of outgrowth of their axons, and the changes in ultrastructure that occur afterwards may be a reflection of a change in the requirements for further growth and maturation of the neurons.

The young retinal ganglion cell may be identified as a classic "neuroblast" by stage 32 on the basis of an increased amount of endoplasmic reticulum and the presence of a large Golgi apparatus, and at stage 33 by the presence of microtubules and microfilaments that appear at the time of axonal outgrowth. In addition to the morphological changes, other criteria may be used to mark the transition from the germinal cell to the neuron, namely the cessation of DNA synthesis and the specification of the central connections of the young retinal ganglion cell shortly after its terminal mitosis (Jacobson, 1967, 1968a, 1968b). By these criteria, the transition from germinal cell to young retinal ganglion cell occurs at stages 29

to 31, that is before we have been able to detect any ultrastructural changes which can be associated with the developmental transition. Thereafter there is a gradual differentiation of the young retinal ganglion cell, until it attains the ultrastructure of an adult cell at about stage 48. We cannot see any advantage in calling the young ganglion cell a neuroblast, when the transition from the germinal cell occurs before ultrastructural changes are evident and when the transition to an adult neuron involves a gradual change in the structure of the cell. We believe that it would be more accurate to regard the young retinal ganglion cell, immediately after cessation of DNA synthesis, as an immature neuron which gradually differentiates into an adult neuron.

Conclusions

The results of this study indicate that:

1. The retinal germinal cells have ultrastructural features of immature cells. However, it seems premature to conclude that they form a structurally homogeneous population, particularly in view of the lack of evidence that they are homogeneous as regards their potential to give rise to all types of retinal cells.

2. There are no ultrastructural criteria by means of which a germinal cell can be distinguished from a so-called "neuroblast", or young neuron.

3. Specification of the central connections of retinal ganglion cells is known from other studies to occur at embryonic stages 30 and 31 but this event is not accompanied by any detectable structural changes in the retinal cells.

4. The first structural differentiation of the retinal ganglion cells is the appearance of an increased amount of endoplasmic reticulum and extensive Golgi zones at stage 32. This is followed by the formation of Nissl bodies, large Golgi zones, microtubules, microfilaments, and the growth of optic axons at stage 33. By stage 35 large zones of Nissl substance appeared in the ganglion cell perikaryon. By stage 48 the retinal ganglion cell bodies have attained the ultrastructural characteristics of the adult state.

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