The fine structure of the reconstructed neural retina of chick embryos

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SUMMARY

The fine structure of reconstructed neural retina formed from dissociated neural retinal cells of 6½-day-old chick embryos on the chorio-allantoic membrane of chick embryos was examined with the electron microscope.

Three nuclear layers (ganglion cell layer, inner and outer nuclear layers) and two fibrous layers (inner and outer plexiform layers) are found within the reconstructed retina.

Both the outer and the inner limiting membranes of the reconstructed structure are constituted from the processes of differentiated Müller cells.

The ganglion cell layer consists of two types of cell, though a typical ganglion cell with axonal process is not observed. Optic nerve fibres are not formed.

Amacrine cells are recognized within the inner nuclear layer.

Differentiation of the inner segment of the photoreceptor cell occurs, but not of the outer segment.

Synaptic structures are recognized in the inner plexiform layer, but not in the outer plexiform layer.

INTRODUCTION

As shown in the previous paper (Fujisawa, 1971), an almost normal neural retinal architecture is established within a pellet of dissociated neural retinal cells of chick embryos implanted on to the chorio-allantoic membrane (CAM). Since this observation was made at the level of the light microscope, the state of differentiation and the extent of reorganization of retinal cells within the reconstructed retina has not yet been reported in detail. So far there have been only a few studies of reconstructed neural structures with the electron microscope (Stefanelli et al. 1967; Sheffield & Moscona, 1970; Adler, 1973).

In the present study observations have been made with the electron microscope on the cytodifferentiation of retinal cells and on cellular organization within the completely reconstructed neural retina formed by grafting the pellets of dissociated retinal cells on to the CAM of chick embryos.

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MATERIAL AND METHODS

Grafting procedures. Centrifuged pellets of singly dissociated neural retinal cells from 6½-day-old chick embryos (stage 30 of Hamburger & Hamilton, 1951) were grafted onto the CAM of chick embryos of the same stage. Details of the procedures for dissociation by trypsin and for grafting were the same as those given in the previous paper (Fujisawa, 1971).

Electron microscopy. 10½ days after grafting, the grafted pellets were taken out and were washed twice in Hanks's solution. Fixation was accomplished with 4% glutaraldehyde in Hanks's solution adjusted to pH 7.2 with 0.1 N sodium hydroxide for 2 h at 4°C. After gentle washing with Hanks's solution, the pellets were post-fixed with 1.0% OsO₄ in the same buffer for 40 min at 4°C and then pre-stained with 2% aqueous uranyl acetate for 60 min. Dehydration was carried out with a graded series of concentrations of ethanol. The material was embedded in Epon 812. Thin sections made with an MY-1 (Sorvall, USA) ultramicrotome were stained with lead citrate according to Reynolds (1963). Observations were carried out with a JEM-7A electron microscope (Japan Electron Optics Laboratory Co., Tokyo).

OBSERVATIONS

Overall structure of the reconstructed neural retina

After 10½ days' development of the pellets on the CAM, structures corresponding to the neural retina were formed as small vesicles on the CAM. These organized structures consisted of three nuclear layers and two fibrous layers (Fig. 1A). The three nuclear layers, from the inner to the outer layers within a vesicle on the CAM, correspond to the outer nuclear layer, the inner nuclear layer and the layer of ganglion cells respectively of a normally developed neural retina of 17-day-old chick embryos, while the inner and outer fibrous layers correspond to the outer and inner plexiform layers respectively. They are easily identifiable by comparing sections of the reconstructed graft (Fig. 1A) with those of the normal retina (Fig. 1B). Between the reconstructed neural retina and the CAM, a sheet of collagen fibres of 1-6 μm thickness is observed, presumably produced by the mesenchymal cells of the CAM (Fig. 1A').

Fig. 1. (A) Overall structure of reconstructed neural retina stained with toluidine blue; bar: 10 μm; (A') fine structure observed by electron microscope of sheet of collagen fibres (col); bar: 1 μm; (B) normally developed retina of 17-day-old chick embryo stained with toluidine blue; bar: 10 μm. onl: outer nuclear layer; inl: inner nuclear layer; gl: layer of ganglion cell; opl: outer plexiform layer; ipl: inner plexiform layer; p: pigment epithelium; CAM: chorio-allantoic membrane.
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**Figure 2**

Fig. 2. (A) Smooth-surfaced endoplasmic reticulum (ser) of the processes of Müller cells (m); bar: 1 μm; arrow: a junction of macula adhaerens diminuta type. (B) Coated vesicle (cv) and coated pits (cp) in the processes; bar: 1 μm. bm: basement membrane; col: sheet of collagen fibres.
Figures 3, 4

Fig. 3. (A) The cell of Type 1 found in ganglion cell layer; bar: 1 μm; (B) the cell of Type 2 (see text); bar: 1 μm. n: nucleus; G: Golgi complex; ser: smooth-surfaced endoplasmic reticulum; rer: rough-surfaced endoplasmic reticulum; ipl: inner plexiform layer; m: cell processes of Müller; col: sheet of collagen fibres.

Fig. 4. (A) Cell of amacrine cell level of inner nuclear layer; bar: 1 μm; (B) bipolar cell level; bar: 1 μm. n: nucleus of amacrine cell; ipl: inner plexiform layer.
Ultrastructure of the ganglion cell layer

The region of reconstructed neural retina adjacent to the CAM is occupied with broad pedal-like cell processes containing many microfilaments, smooth-surfaced endoplasmic reticulum and free ribosomes (Fig. 2A). The cell membranes facing the collagen sheet are covered with a continuous basement membrane (Fig. 2A, B). Coated vesicles and coated pits which are believed to be concerned with an incorporation of proteins (Roth & Porter, 1964) are recognized (Fig. 2B). The perikaryon of this cell process is not usually recognized in this region of reconstructed retina. All of these characteristics are quite similar to the cell processes of Müller which form the inner limiting membrane of fully developed neural retina of normal chick embryos (Meller & Glees, 1965). Meller & Glees (1965) considered that special junctional structures between cell processes of Müller were not formed in the case of the inner limiting membrane of the normally developed retina. In the reconstructed retina, however, junctions of the macula adhaerens diminuta type (Hay, 1968) are often formed (Fig. 2A).

The nuclear layer in this region of reconstructed retina consists of one to two rows of nuclei (Fig. 1A; gl). Electron microscopic observation reveals two types of cells in this region. One (Type 1) is the cell with electron-dense and irregularly shaped nucleus (Fig. 3A), and with the cytoplasm containing rough-surfaced endoplasmic reticulum and a number of free ribosomes, particularly at the periphery, and smooth-surfaced endoplasmic reticulum at the centre. A Golgi complex, mitochondria and lysosomes are also observed. In comparison with the typical ganglion cell, the development of rough-surfaced endoplasmic reticulum of the cell of Type 1 is poor. The other type of cell (Type 2), has an electron-transparent nucleus and with cytoplasm containing a number of polysomes, smooth-surfaced endoplasmic reticulum, mitochondria, microtubules and vacuoles (Fig. 3B). There is very little rough-surfaced endoplasmic reticulum. This type of cell may be identified as the amacrine cell (see later).

No outgrowth of axons or formation of the optic fibre layer has been seen in the reconstructed retina.

Ultrastructure of inner nuclear layer

As shown in Fig. 1A, the region of the inner nuclear layer facing the inner plexiform layer is occupied by large cells whose nucleus and cytoplasm stained lightly with toluidine blue. The corresponding cells are also found in the amacrine cell layer of the normally developed neural retina of 17-day-old chick embryos (compare Fig. 1A with Fig. 1B). As shown in Fig. 4A, the fine structures of these cells are quite similar to those of the cells of Type 2 found in the ganglion cell layer (compare Fig. 4A with Fig. 3B). These structural characteristics are also similar to those of the amacrine cells of young Rhesus monkeys (Dowling & Boycott, 1966).
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The receptor side of the inner nuclear layer consists of many small cells lying perpendicularly to the retinal sheet (Fig. 4B). These cells are similar to cells in the bipolar cell layer of normally developed retina.

Ultrastructure of outer nuclear layer

The cells in this region of a reconstructed retina adhere to each other by an intercellular junction of the zonula adhaerens type (Farquhar & Palade, 1963) and they form a structure corresponding to the outer limiting membrane of a normally developed retina (Fig. 5A). Besides the microvilli belonging to the Müller cells (Meller & Glees, 1965), tongue-like cytoplasmic processes are seen protruding beyond the outer limiting membrane (Fig. 5A). These processes contain, at the apical end, a large number of elongated mitochondria lying parallel to the axis of the processes (Fig. 5A). Such an accumulation of mitochondria may correspond to the ellipsoid of the inner segment of retinal photoreceptor cells. In the proximal region of the processes, a mass of tubular and sac-shaped smooth-surfaced endoplasmic reticulum, without glycogen bodies, is seen (Fig. 5B). Such a structure is assumed to be the immature paraboloid (Meller & Breipohl, 1965). Between the ellipsoid and paraboloid structure, rough-surfaced endoplasmic reticulum, which sometimes shifts to smooth-surfaced endoplasmic reticulum, is seen (Fig. 5B). Oil droplets, which are known to present in the chick retinal cells (Coulombre, 1961), are also seen in the cell processes. They contain an electron-dense material (Fig. 5C).

These structural characteristics of the cell processes are entirely similar to those seen in the inner segment of photoreceptor cells of 16- to 19-day-old chick embryos (Meller & Breipohl, 1965).

Although bearing cilia, and although a pair of centrioles is often observed at the apical end of the inner segment (Fig. 6), the outer segment is not formed within the reconstructed neural retina. On the other hand, a well-developed outer segment is already formed in the retina of 17-day-old chick embryos.

Ultrastructure of inner and outer plexiform layers

A number of cell processes containing both ribbon-like cytoplasmic inclusions, and vesicles of about 200–300 Å (20–30 nm) in diameter are found in the fibrous layer of the reconstructed retina (Fig. 7A, B), corresponding to the inner plexiform layer of a normally developed retina. Such a cell process is very similar to the pre-synapse of bipolar terminal described in the inner plexiform layer of the neural retina of 14-day-old chick embryos (Sheffield & Fischman, 1970). As shown in Fig. 7A and B, the two thickened plasma membranes of cell processes contact the thickened membrane of the pre-synapse, and form the post-synaptic membranes. The ribbon-like structure of the pre-synapse is positioned to point directly between these two post-synaptic membranes. Although in the present observations the identification of these post-synaptic cell processes was impossible, such an intercellular junction may
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be considered to be the developing synaptic contacts of bipolar terminals with ganglion cell dendrites and amacrine cell processes as was found in the primate retinæ (Dowling & Boycott, 1966).

Besides the typical synaptic structures, junctions similar to the macula adhaerens diminuta are found in the inner plexiform layer of the reconstructed neural retina (Fig. 7B). The same type of junction was first seen in the inner plexiform layer of 10-day-old chick embryos and was considered to serve to stabilize intercellular relationships during the development of the plexiform layer (Sheffield & Fischman, 1970).

In the layer corresponding to the outer plexiform layer of the normally developed retina, junctions of the type macula adhaerens diminuta are found, but the typical pre-synaptic structures of photoreceptors and special inter-receptor contacts are not present.

DISCUSSION

The present observations show that the neural retina formed from dissociated immature neural retinal cells of 6½-day-old chick embryos has an almost completely normal architecture not only at the light microscopic level (Fujisawa, 1971) but also at the electron microscopic level.

It seems that Müller cells have important functions for the complete reconstruction of retinal cells. There are many reports on the function of the Müller cells of the retina (reviewed by Cohen, 1963). As in the case of the normally developing retina, Müller cells within the reconstructed retina constitute both the outer and inner limiting membranes as well as the cytoplasmic network, and thus they seem to be essential for maintaining the structural organization of the reconstructed retina (Figs. 2, 5A). As shown in the previous paper (Fujisawa, 1973), the first step of the reconstruction of retinal cells is the formation of rosette-forming aggregates, and all of the cells within a rosette come to be in contact with each other, with a junction of the zonula adhaerens type at its centre (Sheffield & Moscona, 1970). As the Müller cells differentiate, this junctional region is thought to develop into the outer limiting membrane of the reconstructed retina. In the region of the inner limiting membrane, a special junctional structure is formed between cell processes of Müller cells in the

FIGURES 5-7

Fig. 5. (A) Inner segments of photoreceptor cells and microvilli of Müller cells; bar: 1 μm; (B) proximal region of inner segment; bar: 1 μm; (C) oil droplets (o) within an inner segment; bar: 1 μm. ml: mitochondria; rer: rough-surfaced endoplasmic reticulum; p: paraboloidal structure; arrows: junctions of zonula adhaerens type.

Fig. 6. Cilia with a pair of centrioles at apical portion of inner segment of photoreceptor cell; bar: 1 μm. arrow: a junction of zonula adhaerens type.

Fig. 7. (A, B) Synaptic structures (S) and a junction of macula adhaerens diminuta type (arrow) in inner plexiform layer; bar: 0.5 μm.
reconstructed retina (Fig. 2A), whereas such a structure is not formed in the normally developing retina. Such a structure seems, therefore, to be necessary to permit the reaggregation and rearrangement of dissociated retinal cells. As shown in Fig. 2B, Müller cells in a reconstructed retina possess a number of coated vesicles and coated pits. Because the capillaries and other blood vessels do not penetrate into the reconstructed retina, the incorporation of nutrient must be mediated by the Müller cells.

Typical ganglion cells are not recognized in the ganglion cell layer of the reconstructed retina. As shown in Fig. 3A, the cell of Type 1 found in the ganglion cell layer is in several structural characteristics different from a normal ganglion cell, although they are similar in some respects. At stage 30 of development, when retinae were removed for the present experiment, optic nerve fibres have already been formed (Rogers, 1957), so optic nerve fibres must be cut when the neural retina is dissociated. The absence of a target organ for the optic nerve, such as the optic tectum, within a grafted pellet, may inhibit the regeneration of optic nerve and probably causes the degeneration of ganglion cells. If this is true, the cell of Type 1 may be considered to be a degenerating ganglion cell. On the other hand, the possibility that the cell of Type 1 is the other kind of cell still remains. Further analyses are necessary to clarify the problems of the differentiation of ganglion cells, and the formation of optic nerve fibres in vitro.

Outer segments of the photoreceptors are formed in the control retina of 17-day-old chick embryos, but not in the reconstructed retina. An absence of the outer segments in a reconstructed retina may be partly due to the overall retardation of development, and partly to the absence of pigment epithelium. It has been reported that a close apposition to the pigment epithelium (Kroll & Machemer, 1969), as well as the presence of vitamin A (Dowling & Gibbons, 1961), seems to be necessary for completion of the outer segments of photoreceptors. If this is correct, a mixed population of neural retinal cell and pigment epithelium, if cultured under appropriate conditions, would be expected to form the outer segments of the photoreceptor cells.

In the inner plexiform layer of the reconstructed neural retina, pre-synaptic structures of bipolar terminals are formed. But in the outer plexiform layer pre-synaptic structures of photoreceptors cannot be observed. It has been reported that well-developed pre-synaptic structures of photoreceptors are formed after cultivating the reaggregated retinal cells of chick embryos for 25 days (Stefanelli et al. 1967). In the present experiments, culturing on the CAM was done for 10 days. Probably, prolonged cultivation may be necessary for the development of the synaptic structures in the outer plexiform layer.

Ideas about the role of the macula adhaerens diminuta found in the developing plexiform layer are contradictory. Meller (1964) suggests that the increase of electron density of pre- and post-synaptic membranes precedes the aggregation of synaptic vesicles. On the other hand, Sheffield & Fischman (1970) consider that the macula adhaerens diminuta is the stabilizing structure. In the
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present observations, it is not possible to decide whether the macula adhaerens diminuta is the developing synaptic structure or not.

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REFERENCES


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