An electron-microscope study of centrioles in differentiating motor neuroblasts

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Mature neurons with centrioles were first described at the end of the nineteenth century and have been observed in many animals (see Cajal, 1911; Ariëns Kappers, Huber & Crosby, 1936). As mitosis rarely, if ever, occurs after morphological differentiation of the neuroblast begins, the function of the centrioles in nerve cells posed a problem which has yet to be resolved. Held (1909) described centrosomes in differentiating neuroblasts as being associated with the 'fibrillogenous zone', which suggests a role for the centriole in the differentiation of neurofibrils.

Recently, electron-microscopic observations have refocused attention on the possibility of morphological and functional association between the centrioles of nerve cells and the fibrillar elements, especially neurotubules, which appear to be similar in fine structure to microtubules of other types of cells and to spindle tubules of the mitotic apparatus. Gonatas & Robbins (1965), studying retinal cells in the 8-day chick embryo, and Tennyson (1965), studying the differentiation of spinal ganglion cells in the rabbit embryo, have suggested that neurotubules and other microtubules may be products of centriolar activity, at least in animal cells. On the other hand, observations of neurofilaments and neurotubules during the initial phases of differentiation in the chick-embryo motor neuroblast have raised questions as to whether the centrioles play any direct role in the formation of neurotubules, particularly those which appear in the axon as it begins to develop (Lyser, 1968).

The present paper reports further study of the problem in the spinal cord of 3-day chick embryos. This stage was chosen for investigation of possible association between centrioles and neurotubules in the developing neuron since numerous cells at various early stages of morphological differentiation can be seen. Cells at stages from neural epithelial cell through monopolar neuroblast were studied.

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MATERIALS AND METHODS

The chick embryos used in this study were at developmental stages 19 and 20 of Hamburger & Hamilton (1951). Embryos were incubated in the laboratory at 38° C. Lengths of neural tube from the level of the wing bud or leg bud were dissected as described previously (Lyser, 1964). Pieces included spinal cord and parts of adjacent somites, notochord, ectoderm, and endoderm.

The tissue was fixed in 3 % glutaraldehyde in 0.05 M phosphate buffer with 0.0015 M-CaCl₂ (Sabatini, Bensch & Barnett, 1963; Tilney & Porter, 1965) for 45 min to 1 ½ h, rinsed in phosphate buffer, post-fixed in 2 % OsO₄ in 0.1 M phosphate buffer or in Tyrode solution for 30–45 min, dehydrated in ethanol, and embedded in Maraglas (Spurlock, Kattine & Freeman, 1963) or Epon 812 (Luft, 1961). All steps were carried out at room temperature. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with glass or diamond knives. Thick sections (0.5-1 μ), stained with toluidine blue and pyronine (Ito & Winchester, 1963) or viewed unstained by phase microscopy, were used for locating the desired region. Some sections for electron microscopy were examined without staining, some were stained with uranyl acetate (Watson, 1958), or uranyl acetate followed by lead citrate (Coggeshall, 1965). They were viewed in an RCA EMU-3H electron microscope.

The neural tube was sectioned transversely in all cases. Blocks were trimmed so that thin sections included the ventral lateral area of at least one side of the spinal cord from the neurocoel to the outer edge. Survey micrographs covering considerable areas of the ventral lateral part of the neural tube were taken at low magnifications (× 1900–3100) for overall orientation and confirmation of the relationships of the cells of interest. Original plates of cells with centrioles were taken at magnifications up to × 24000 and further enlarged photographically. Sections from four embryos were studied; micrographs include twenty-six neuroblast cells with centrioles visible in one or more sections.

EXPLANATION OF PLATES

All figures are electron micrographs of cells from transverse sections of the ventral lateral region of the spinal cord of 3-day chick embryos. Plates 1 to 2, fig. B, and Plates 3-5, uranyl acetate stain; Plate 2, fig. C, unstained.

PLATE 1

Fig. A. Apical ends of ciliated neural epithelial cells. In the cell at the right the section cuts longitudinally through the base of the cilium (ci) and associated centrioles (c₁, c₂); the apical centriole (c₁) is the basal body. The plasma membrane is invaginated at the base of the cilium. In the cell at the left, the section includes the edge of the cilium and passes to the side of the centrioles but includes a dense body (d), probably associated with the centrioles. Microtubules (mt) are associated with centrioles or a dense body. nc, neurocoel. × 30000.

Fig. B. Apical ends of neural epithelial cells. The usual deposition of centrioles near the edge of the neurocoel (nc) can be seen, including one pair associated with a cilium (ci). Another pair of centrioles, with membranes and tubules like those at the bases of cilia at the end of one centriole (arrows), is apparently moving away from the edge of the neurocoel. × 15000.
RESULTS

In describing cells of the neural tube terms indicating direction are used as in previous papers (Lyser, 1964, 1968). For neural epithelial cells, the part of the cell toward the outer edge of the neural tube is considered as the basal end and that toward the neurocoel (inner edge of the neural tube) as the apical end. As the neuroblast differentiates and the organization of the cell changes, these terms no longer apply. In describing motor neuroblasts, 'lateral,' 'peripheral' and 'distal' denote orientation in the embryo, hence toward the outer edge of the neural tube; 'medial' and 'proximal,' toward the neurocoel (inner edge of the neural tube). In all cases, 'inner' and 'central' indicate toward the neurocoel; 'outer,' toward the outer edge of the neural tube.

By the third day of incubation, three zones can be recognized in the wall of the neural tube: the inner zone of neural epithelial cells, the mantle layer of differentiating neuroblasts, and the peripheral marginal layer of nerve fibers. Distinct ventral roots are present.

The neural epithelial cells are a proliferating population which give rise to neuroblasts and later to glial cells. Interphase cells form a columnar epithelium with nuclei at various levels; cells undergoing division round up near the neurocoel (F. C. Sauer, 1935a, b; M. E. Sauer & Chittenden, 1959; M. E. Sauer & Walker, 1959; Sidman, Miale & Feder, 1959; Fujita, 1962; Källén & Valmin, 1963; Martin & Langman, 1965). The first motor neuroblasts to differentiate are located among neural epithelial cells. By the third day the more advanced motor neuroblasts move peripherally and form a mantle layer in the ventral lateral part of the neural tube. Motor neuroblasts have been identified in electron micrographs in this study on the basis of their position and relationships in the neural tube. Light-microscopic observations of silver-stained sections, studies with \(^{3}\text{H}\) thymidine, in which virtually all neural epithelial cells but no differentiating neuroblasts become labelled, and the general fine structure indicate that most or all cells in the ventral lateral mantle zone are differentiating motor neuroblasts with axons. In electron micrographs the base of the axon can

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**PLATE 2**

Fig. A. Neuroblast with centrioles (c) located in the inner end of the cell. This cell is just dorsal to the ventral root; the top of the figure is toward the neurocoel, the bottom toward the outer edge of the neural tube. A second centriole and some associated membranes can be seen in sections serial to this one. g, Golgi complex; n, nucleus. × 9900.

Fig. B. Higher magnification of centrioles of the same cell, from a section serial to that shown in fig. A. Some microtubules (mt) are adjacent to the centrioles, others are scattered in the cytoplasm. × 38000.

Fig. C. Neuroblast with a centriole (c) medial to the nucleus (n), and the axon (a) arising from the opposite—lateral—side of the cell. This neuroblast is located in the 3rd layer of cells from the outer edge opposite the ventral root. × 12000.
be seen in a few of these neuroblasts. The less advanced motor neuroblasts medial to the outer mantle zone cannot be definitely identified in electron micrographs unless the axon is included in the section.

The interphase neural epithelial cells of 3-day embryos and earlier stages have a few microtubules scattered throughout the cytoplasm and numerous microtubules in the apical cytoplasm. The earliest neuroblasts with processes observed by this author have a few scattered microtubules in the cell body and numerous microtubules and fine filaments in the axons. The microtubules of the neural epithelial cells, those of the neuroblasts, which are neurotubules, and the spindle tubules of the dividing cells all show similarities to each other and to microtubules in other types of cells.

**Neural epithelial cells**

In the majority of the interphase neural epithelial cells the centrioles were located in pairs at the apical ends of the cells. In most sections a few cilia arising from these centrioles could be seen (Plate 1, figs. A, B). The cilia arose in invaginations in the free surface of the plasma membrane. The basal body, or apical centriole, was just below the bottom of this invagination, and in sections which cut the centriol and cilium longitudinally, the plasma membrane turned from the lining of the invagination on to the cilium at the apical end of the centriole (cilium at right, Plate 1, fig. A). Many microtubules in the vicinity of the centrioles appeared to end close to the centrioles or at an electron-dense area adjacent to a centriole (Plate 1, fig. 1), presumably representing the clavate body described by Gonatas & Robbins (1965). These microtubules extended from the centrioles either (a) away from the neurocoel more or less parallel to the long axis of the cell, or (b) across the apical end of the cell, more or less parallel to the edge of the neurocoel. Occasionally, a centriole or a pair of centrioles was seen in the apical end of a cell a few microns away from the neurocoel (Plate 1, fig. B). Membrane profiles and tubular elements like those at the base of a cilium were sometimes associated with these centrioles (Plate 1, fig. B, arrows).

**Motor neuroblasts**

In differentiating motor neuroblasts, centrioles were found at various locations in the cell. Some centrioles were located at the base of the axon and axon neurotubules were closely associated with them. However, arrangements in

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**Plate 3**

Fig. A. Neuroblast with a centriole (c) at the side of (ventral to) the nucleus. The base of the axon (a) extends laterally and contains a number of microtubules. Golgi complex membranes (g) are visible in the lateral part of the cell and near the centriole. \( \times 9900 \).

Fig. B. Higher magnification of the centriole (c) of the cell in fig. A and microtubules (mt) near the centriole. n, Nucleus. \( \times 44000 \).
other neuroblasts suggest that there is no essential direct relationship between
the centrioles and the axon or its neurotubules.

Centrioles medial to the nucleus were seen in one neuroblast with the base of
the axon included in the same section, and in several other cells which were
identified as neuroblasts with axons on the basis of their position in the neural
tube (Plate 2, figs. A–C). These cells were in the second layer of cells from the
outer edge of the neural tube or a little farther in, and appeared to be slightly
less differentiated than the most advanced 3-day motor neuroblasts. The cen-
trioles were adjacent to the nucleus (Plate 2, fig. C) or closer to the inner end of
the cell (Plate 2, fig. A).

In some sections neurotubules terminated near the centrioles and extended in
various directions from them (Plate 2, fig. B). It was not possible to determine
the length of any individual neurotubule but no connexions were evident be-
tween neurotubules near the centriole and those in the axon or most of those
scattered in other parts of the perikaryon. Occasionally a series of fairly long
neurotubules or segments of tubules were seen extending from the region of the
centriole along the dorsal or ventral side of the nucleus to the lateral end of the
cell.

In other neuroblasts centrioles were located at the dorsal or ventral side of the
nucleus, between the inner and outer ends of the cell (Plate 3, fig. A), or in the
lateral part of the cell body at some distance from the base of the axon. These
centrioles were close to the nucleus or anywhere between the nuclear envelope
and the plasma membrane. Some neurotubules in the adjacent cytoplasm ended
close to the centrioles, extending away from them in various directions (Plate 3,
fig. B). Again, no direct connexions with neurotubules of the axon were observed.

The centrioles of other neuroblasts were found at the base of the axon or in
the narrow outer end of the cell from which the axon arose (Plate 4, figs. A, B).
Neurotubules were conspicuous in this part of the neuroblast. Some of them
were closely associated with the centrioles.

Neuroblast centrioles, whether medial or lateral to the nucleus, or in an inter-
mediate position, were present in pairs, with the centrioles at an obtuse angle to

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Plate 4

Fig. A. Neuroblast (nb) with centrioles (c) at the base of the axon (a). Marginal zone (mz) and
outer edge of the neural tube are at the bottom of the figure. × 3700.

Fig. B. Higher magnification of centrioles (c) of the neuroblast shown in fig. A from a section
serial to that one. A number of microtubules—neurotubules—(mt) are present in this part
of the cell as well as further distally. × 38000.

Fig. C. Neuroblast centrioles with a membrane which is apparently a flat vesicle at the end of
one (arrow). g, Golgi membranes; mt, microtubules. This cell is located at the outer edge of
the mantle zone just ventral to the ventral root; the centrioles are ventral to the nucleus.
× 44000.

Fig. D. The same centrioles as shown in fig. C from a section serial to that one. Associated
membrane still has the appearance of a flat vesicle (arrow). × 26000.
one another. Sections cutting a centriole transversely or nearly transversely included only one centriole; when the plane of section was longitudinal or oblique, one or two centrioles were included in the section. More than two were not encountered in one neuroblast. When two centrioles were seen, they were adjacent to each other and of approximately the same size. No profiles of small centrioles perpendicular to larger ones were seen.

Neuroblast centrioles were frequently near membranes of the Golgi complex (Plate 4, fig. C) or encircled by them. Ribosomes and polysomes were seen throughout the cytoplasm of the cell body and immediately around the centrioles. Granular endoplasmic reticulum, small cytoplasmic vesicles, or mitochondria were also seen close to the centrioles in some instances.

In some neuroblasts membrane profiles resembling the invaginations of the plasma membranes at the base of the cilia of neural epithelial cells were seen at the end of one of the centrioles. Tubules like those of a cilium sometimes extended from the end of the centriole where the membrane was. Complete cilia were not seen in most neuroblasts; however, clearly visible cilia were found in three cells. Two are shown in Plate 5. The centrioles and cilia were lateral to the nucleus in these cells. One cilium extended out from the dorsal lateral edge of the cell (Plate 5, fig. A, nb1). A profile of cytoplasmic membrane surrounded the other cilium (Plate 5, fig. A, nb2). In other cells a flat membrane profile was seen at the end of one centriole (Plate 4, figs. C, D). The section in Plate 4, fig. C, cut this centriole longitudinally near the center; the membrane also had the appearance of a flat vesicle in another section near the edge of the centriole (Plate 4, fig. D). This suggests that the membrane may in fact have been a flat vesicle in three dimensions, resembling the flat vesicles observed at an early stage in the formation of cilia in fibroblasts and smooth muscle cells (Sorokin, 1962). Such membranes were not seen to be associated with some longitudinally sectioned neuroblast centrioles, but it could not be concluded definitely that none were present, since in some other cells similar membranes could be seen in one longitudinal section but not in a section serial to it.

PLATE 5

Fig. A. Part of the ventral mantle zone, including two neuroblasts (nb1, nb2) with cilia (ci) and one with a centriole (c) lateral to the nucleus (nucleus not included in figure). The top of the figure is medial, the bottom lateral. The base of the cilium of nb2 and the basal body centriole are included in a section serial to this one (fig. C). × 8800.

Fig. B. Higher magnification of cilium (ci) and associated centrioles (c) of nb1, fig. A. Plasma membrane is invaginated around the base of the cilium as in neural epithelial cells (arrows). × 35000.

Fig. C. Base of the cilium (ci) of nb2 from a section serial to that shown in fig. A. It is not known whether the vacuole into which the cilium appears to project is continuous with the plasma membrane. c, Basal body centriole. × 35000.
DISCUSSION

According to Held (1909), centrioles in differentiating neuroblasts are found in the 'fibrillogenous zone', lying first at the point of exit of the axon, and later shifting to the base of the principal dendrite. These observations suggested that there is a causal developmental relationship between the centrioles and the differentiation of neurofibrils (neurotubules) as well as nerve-cell processes. Electron micrographs have shown close association between centrioles and neurotubules in nerve cells in embryos (Gonatas & Robbins, 1965; Tennyson, 1965), including a centriole in the base of the process of an amacrine cell in the retina of the 8-day chick (Gonatas & Robbins, 1965).

However, the above observations do not definitely establish a causal relationship between centrioles and the formation of neurites or the differentiation of neurotubules (neurofibrils). The developing retinal cells studied by Gonatas & Robbins were well past the initial stages of neurite outgrowth, and in some cells the centrioles were fairly near the nucleus (ganglion cell shown in fig. 3b, Gonatas & Robbins, 1965). In rabbit spinal ganglion neuroblasts at the bipolar stage, neurotubules extend from the region of the centriole, located in the vicinity of the Golgi complex in the center part of the cell, toward the ends of the cell. Numerous neurotubules are present in the ends of the cell, which are the bases of the processes, and in the neurites (Tennyson, 1965). Hence, neurotubules can be seen from the centriole to the neurites, but the centriole is not in the immediate area of either neurite.

If the centrioles of the neuroblasts were actually involved in the initiation of the outgrowth of the neurite and the formation of its neurotubules, one would expect to find motor neuroblast centrioles at the base of the axon when it is growing out, especially at the initial stages of outgrowth. No such preferential location was observed in early motor neuroblasts. Instead, centrioles were found in various locations in the cell. Particularly to be noted are those centrioles medial to the nucleus, where the centriole and the axon are at opposite sides of the cell (Plate 2, fig. C). From this evidence it is concluded that in motor neuroblasts the centriole is not directly involved in the formation of the axon or the organization of its neurotubules.

On the basis of the close association observed in rabbit spinal ganglion cells between some neurotubules and the centrioles which are in the Golgi region, Tennyson (1965) suggested that the first neurotubules are formed from the Golgi–centriolar complex, and could move into the neurite from there. Associations between neurotubules and centrioles in the Golgi region are also seen in motor neuroblasts, and could be interpreted in the same way. On the other hand, microtubules formed without intervention of centrioles might become associated secondarily with them when in their immediate vicinity. Further investigation of this aspect of the problem will be carried out using organ culture methods (Lyser, 1966a).
The pattern of the location of centrioles in neuroblasts may be related to the sequence of changes in cell shape and organization during the initial phases of differentiation. Windle and co-workers have described the sequence of changes in the earliest spinal cord motor neuroblasts from observations of silver-stained material. These neuroblasts are first recognized as elongated cells which become 'primitive bipolar' neuroblasts as the axon is formed, then piriform or monopolar neuroblasts as the inner end is withdrawn from the neurocoel, and later multipolar as the dendrites form (Windle & Austin, 1936; Windle & Baxter, 1936). Previous electron-microscopic observations of differentiating motor neuroblasts (Lyser, 1964, 1968) and light-microscopic observations of early differentiation in the chick brain (Lyser, 1966b) are consistent with this interpretation, as are observations reported in this paper. Centrioles, such as those shown in Plate 1, fig. B, which are in the apical ends of cells at a little distance from the neurocoel, could be in the process of being withdrawn toward the main part of the cell body of the early neuroblast, though they could also be centrioles of neural epithelial cells changing their position in preparation for mitosis (Allenspach & Roth, 1967). In neuroblasts the centrioles and Golgi complex appear to change their position as the cell differentiates morphologically. The centrioles and Golgi complex are found in the apical ends of the neural epithelial cells. In early neuroblasts these organelles are still medial to the nucleus. In other neuroblasts, located farther peripherally or at slightly later stages of differentiation, the centrioles and Golgi complex are found at the side of the nucleus or in the lateral part of the perikaryon.

A somewhat different interpretation of early neuroblast differentiation has been given by Langman, Guerrant & Freeman (1966). These workers observed a small number of mitotic figures oriented with the spindle perpendicular, rather than parallel, to the edge of the neurocoel. They concluded that cells that begin to differentiate into neuroblasts are the outer daughter cells which are pushed outward away from the neurocoel at the end of the division. In this event the centrioles should be in the outer end of the outer daughter cell after the completion of the division. One might expect that they would remain in this region of the cell, and would not expect to see centrioles medial to the nucleus in early neuroblasts. Therefore, observations of early motor neuroblasts with centrioles medial to the nucleus seem to be more consistent with the interpretation that early neuroblasts begin to differentiate morphologically from cells which have returned to the epithelial configuration after the last division than with the interpretation that they differentiate immediately from newly formed daughter cells.

In this study neuroblast centrioles were observed to be associated with cilia in a few cells, but the significance of this observation is not clear. It is not known how many of the neuroblasts are ciliated, since without extensive serial sections it cannot be decided whether the membranes and tubules seen in other neuroblasts were oblique sections of the bases of relatively well-formed cilia, or
whether there were only remnants of cilia in most cells. One possibility is that cilia in these differentiating neuroblasts may have been retained from the preceding stage of development, since neural epithelial cells are ciliated. Configurations with the appearance of the base of a cilium in the apical ends of cells at a little distance from the neurocoel, as well as in the cell bodies of neuroblast cells, are consistent with such an interpretation.

However, ciliated cells may be more widespread than generally observed. Cilia have been seen in cells which are not ciliated at preceding stages of development; for example, fibroblasts and smooth muscle cells (Sorokin, 1962). Among the nerve cells in which cilia have been seen are granular cells in the hippocampal region of the cerebral cortex of adult rats (Dahl, 1963), spinal cori (Duncan, quoted by Dahl, 1963), corpus striatum (Mugaini, quoted by Dahl, 1963), neurosecretory cells in the preoptic nucleus of the goldfish (Palay, 1961), autonomic ganglion neurons, as well as Schwann cells, of young adult rats (Grillo & Palay, 1963), and a Purkinje cell of a monkey fetus (Kornguth, Anderson & Scott, 1967). Although a sensory function has been suggested for these and other presumably non-motile cilia, no conclusive evidence has been found to confirm this suggestion (Barnes, 1961; Dahl, 1963).

SUMMARY

1. The relationships between centrioles and microtubules and axons of neuroblasts at early stages of differentiation have been examined in the spinal cord of 3-day chick embryos fixed in glutaraldehyde. Motor neuroblasts as well as neural epithelial cells, which give rise to neuroblasts, were studied.

2. In interphase neural epithelial cells a pair of centrioles is located at the apical edge of the cell. The apical centriole is the basal body of a cilium. Microtubules are prominent in the apical ends of many of these cells, and some are associated with the centrioles.

3. In motor neuroblasts with axons, centrioles also occur in pairs and are located in the inner (medial) end of the cell, at the side of the nucleus, or in the outer (lateral) part of the cell. Microtubules (neurotubules) are scattered in the cell body and are numerous in the axon. Some microtubules are associated with neuroblast centrioles whatever their location in the cell, but no continuity has been seen between axon neurotubules and those near centrioles which are located in other regions of the cell.

4. These observations indicate that the centrioles are not essentially involved in the initiation of axon outgrowth or in the organization of the neurotubules of the neurite.

5. In a few cases neuroblasts with cilia have been encountered; one of the pair of centrioles forms the basal body as in neural epithelial cells. The significance of these neuroblast cilia is not clear.
RÉSUMÉ

Etude des centrioles en microscopie électronique, dans des neuroblastes moteurs en cours de différenciation

1. Les relations entre les centrioles et microtubules et les axones des neuroblastes aux stades précoces de leur différenciation ont été examinées dans la moëlle épinière d'embryons de poulet de 3 jours fixés à la glutaraldéhyde. On a étudié les neuroblastes moteurs, aussi bien que les cellules épithéliales neurales qui donnent naissance aux neuroblastes.

2. Dans les cellules épithéliales neurales en interphase, une paire de centrioles est située au bord apical de la cellule. Le centriole apical est le corps basal d'un cil. Des microtubules font saillie dans les extrémités apicales de bon nombre de ces cellules et quelques-uns sont associés avec les centrioles.

3. Dans les neuroblastes moteurs pourvus d'axones, des centrioles se trouvent aussi par paires et sont localisés dans la partie la plus interne (médiane) de la cellule, à côté du noyau, ou dans la partie la plus externe (latérale) de la cellule. Les microtubules — neurotubules — sont dispersés dans le corps cellulaire et sont nombreux dans l'axone. Quelques microtubules sont associés aux centrioles des neuroblastes quelle que soit leur localisation dans la cellule mais on n'a pas vu de continuité entre les neurotubules de l'axone et ceux qui se trouvent près de centrioles localisés dans d'autres régions de la cellule.

4. Ces observations indiquent que les centrioles ne sont pas essentiellement impliqués dans l'initiation de la croissance de l'axone ou dans l'organisation des neurotubules du neurite.

4. Dans quelques cas, on a rencontré des neuroblastes avec des cils; une des paires de centrioles forme le corps basal comme dans les cellules épithéliales neurales. La signification de ces cils neuroblastiques n'est pas claire.

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