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Ultrastructural Observations on Closure of the Neural Tube in the Mouse

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Summary. The fusion of the neural walls in the cephalic part of mouse embryos varying in age from 9 to 20 somites was examined with the electron microscope. In the rhombencephalic region the rim of the neural wall was formed from outside inward by ectodermal surface cells, a row of flattened cells without surface projections and neuroepithelial cells. At the junction of the surface ectoderm and the flat cells were seen large projections containing a cytoplasmic matrix without organelles and previously referred to as "ruffles". The initial contact between the walls was made by the large cytoplasmic arms and numerous finger-like projections interdigitating with similar projections from the opposite wall. The projections originated from the surface ectoderm and possibly neural crest cells. During further fusion the surface ectoderm cells formed dense membrane specializations, thus establishing a firm contact.

The initial contact in the mesencephalon was formed by extensions from the surface ectoderm and was followed by the formation of specialized membrane junctions, as seen between the surface ectoderm in the rhombencephalon. The neuroepithelial cells facing the gap between the neural walls with their apical ends made contact with the cells from the opposing wall by numerous finger-like projections but membrane specializations failed to develop.

The closing mechanism in the prosencephalon and anterior neuropore regions differed from the previous areas in that the initial contact was established by the neuroepithelial cells. Only after this contact had been formed did the surface ectoderm cells close the gap. In contrast with the other areas many phagocytosed particles were seen in the prosencephalon and

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in the region of the anterior neuropore. Many particles from degenerated cells were found inside healthy surrounding cells. Some of these particles contained nuclear material and cytoplasmic organelles.

Key words: Closure – Development – Mammalian embryo – Neural tube – Ultrastructure.

Introduction

From recent studies on closure of the neural tube in mouse embryos it has become evident that fusion of the neural walls does not proceed from the rhombencephalon to the anterior neuropore in a zipper-like fashion. In the rhombencephalon and prosencephalon fusion of the neural walls proceeds simultaneously (Geelen and Langman, 1977). Similar observations were made in the rat by Edwards (1968) and Christie (1964) and in the hamster by Keyser (1972) and Shenefelt (1972). With scanning electron microscopy Waterman (1976) similarly demonstrated that fusion occurs simultaneously in the hindbrain and in the forebrain.

Examining the cells involved in the initial contact between the opposing neural walls, it was found that in the rhombencephalon surface ectoderm and probably neural crest cells were the first ones to make contact; in the prosencephalon and particularly in the region of the anterior neuropore, the neuroepithelial cells were involved in the fusion process; in the mesencephalon, the first contact was mainly made by surface ectoderm cells (Geelen and Langman, 1977).

A number of investigators have examined the neurulation process in amphibian and chick embryos with the scanning electron microscope (Tarin, 1971; Gouda, 1974; Barson and Portch, 1974; and Portch and Barson, 1974), but little work has been performed on the closure process of the neural tube in the mammalian embryo (Waterman, 1975a, b; 1976). When Waterman studied the cellular morphology along the rims of the neural folds before and after fusion in hamster and mouse embryos, a narrow band of flattened cells was found between the surface ectoderm and the neural ectoderm regions. In the mouse numerous membranous "ruffles" were seen at the junction of the surface ectoderm and the so-called flat cells and these ruffles were thought to establish the first contact between the opposing folds. Since scanning electronmicroscopy provides mainly surface characteristics, it was difficult to determine how contact between the cells in the deeper layers was established.

Only few studies have been performed on closure of the neural tube with transmission electronmicroscopy (Schroeder, 1970; Löfberg, 1974; Bancroft and Bellairs, 1975; Schlüter, 1973). Schlüter examined the cells of the neural tube in the mouse embryo before and after closure, but focused his attention on the role of cell death. Bancroft and Bellairs (1975) noted in the chick embryo that at the time of fusion some threads, probably formed by the fusion of two projections, were connecting the opposing folds. Microvilli and other cytoplasmic processes appeared to be touching cells in the opposite neural fold, but cell junctions were not observed.

This study was undertaken to examine with the transmission electronmicroscope the manner by which cellular contact between the approaching folds is established and whether membrane specializations are formed.

Materials and Methods

Female ICR mice from Flow Laboratories (Dublin, Va.) were mated from 9–12 A.M. and subsequently examined for the presence of vaginal plugs. The day on which a plug was found, was considered as day 1 of gestation.

The pregnant females were sacrificed on day 10 at 10 A.M. It is known that at this stage of development embryos at different stages of neural tube closure can be found in one litter (Geelen and Langman, 1977). Therefore this stage of development is particularly suitable to examine the first contact and subsequent fusion between the opposing walls. After the embryos were removed from the uterus and membranes, they were fixed for one hour in modified Karnovsky (1965) solution (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3). The tissue was then post-fixed for one hour in 0.1 M cacodylate buffer containing 1% OsO_4 and after dehydration in ethanol embedded in Epon 812. Embryos in which the prosencephalon, mesencephalon and rhombencephalon were partly closed were selected for sectioning. After orientation they were cut at 1 µm on a Sorvall, Porter-Blum MT2 ultramicrotome. As soon as one of the fusion areas between the neural walls was reached, 300–600 Å sections were cut with a diamond knife. The thin sections were subsequently stained with uranyl acetate and lead citrate and examined with a RCA EMU 3 electron-microscope. In this manner sections of the fusion process in the rhombencephalon, mesencephalon, prosencephalon and the anterior neuropore were obtained.

Results

Closure of the Rhombencephalon. In the rhombencephalon region a single layer of ectoderm cells covered the outer surface of the embryo up to the rim of the neural wall (Fig. 1). These surface ectoderm (SE) cells were characterized by microvilli projecting from the surface. Immediately over the tip of the neural wall and facing the lumen of the neural groove one or sometimes two flat attenuated epithelial cells (FE) were seen (Figs. 1 and 2). Over most of their surface they had few if any cytoplasmic projections extending into the lumen of the groove. At the junction of the flat epithelial cell with the surface ectoderm, however, were always noted thick projections (R) containing cytoplasmic matrix without organelles (Figs. 1 and 2). These unusually large projections (in SEM studies referred to as ruffles) were sometimes cup-shaped, but more frequently had the appearance of multiple fingerlike extensions. They were greatly different from the microvilli on the surface ectoderm. In some instances the ruffles were seen to originate from the flat epithelial cells (Fig. 2) and in other instances from the surface ectoderm cells (Fig. 1). Deeper in the neural groove the wall was formed by the apical ends of the neuroepithelial cells (NE), which were always connected to each other by typical tight junctions. Cytoplasmic blebs containing ribosomes, mitochondria and rough endoplasmic reticulum protruded from the apical surface and formed the luminal surface (Fig. 3).

In sections closer to the fusion area long cytoplasmic arms extended into the gap between the two opposing rims (Fig. 4). The actual contact was made by numerous finger-like projections interdigitating with similar projections from

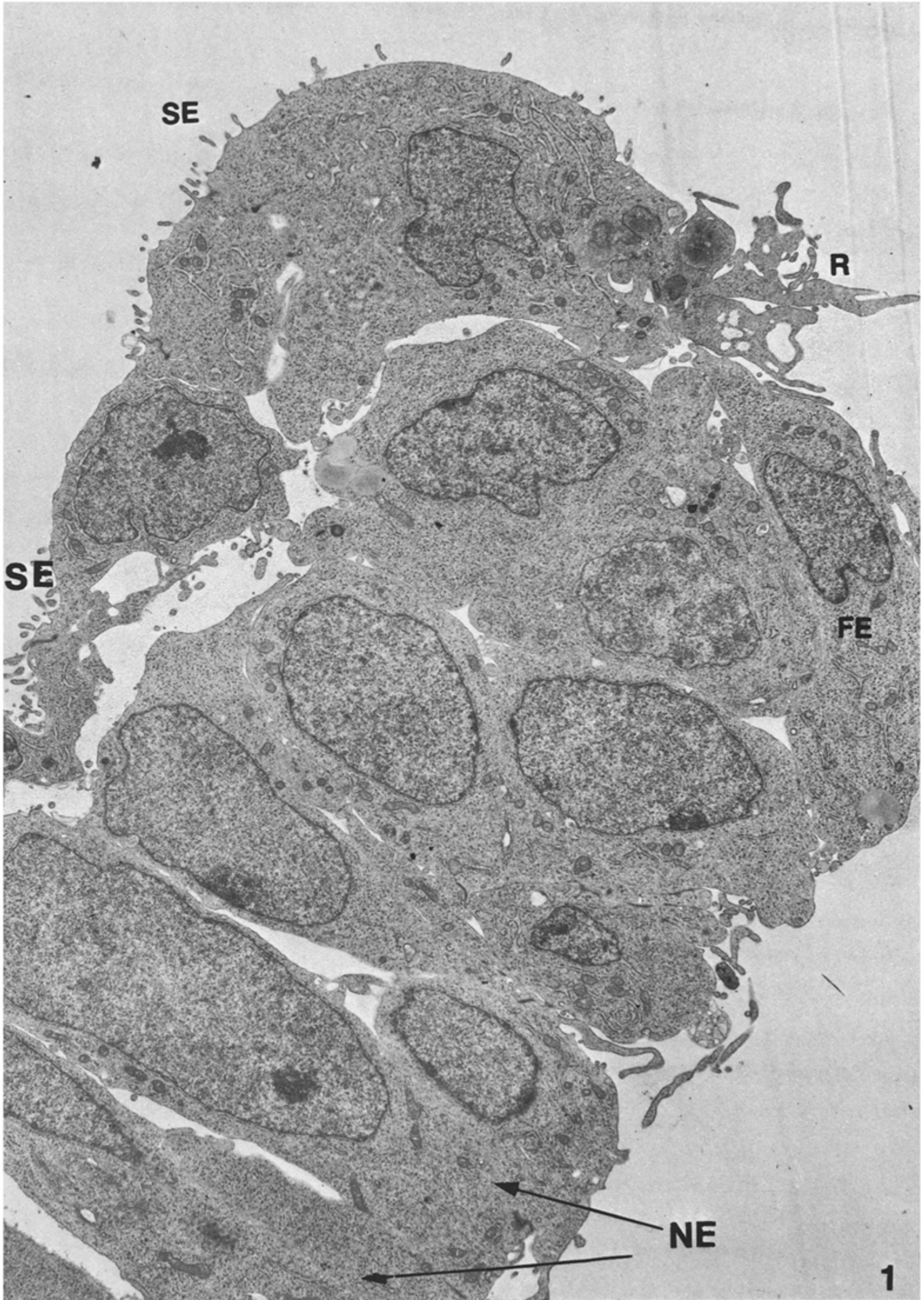


Fig. 1. Transverse section through the rhombencephalon prior to fusion. The cellular components of the rim of one of the neural walls are shown. Note the surface ectoderm (*SE*), the flat epithelial (*FE*) cell and the neuroepithelial (*NE*) cells. A prominent cytoplasmic extension, a so-called ruffle (*R*) is present at the junction of the surface ectoderm and the flat epithelial cell. 4,500 ×

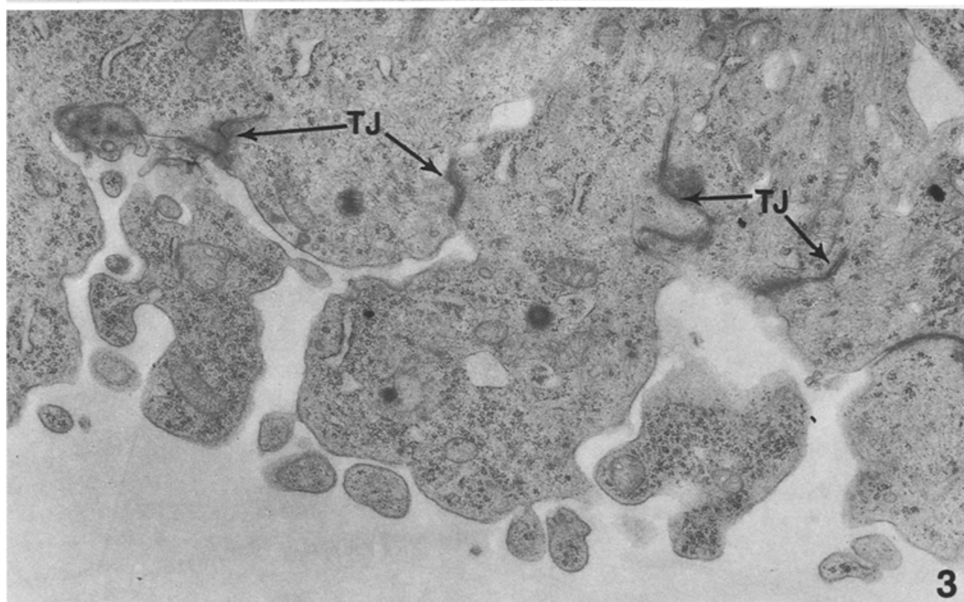


Fig. 2. Rim of neural wall in rhombencephalic region showing surface ectoderm (*SE*) cells, a flat epithelial cell (*FE*) and neuroepithelial cells (*NE*). Note that the cytoplasmic extension, referred to as ruffle (*R*), seems to originate from the flat cell. 4,600 ×

Fig. 3. Cytoplasmic blebs protruding from the apical ends of the neuroepithelial cells. The protrusions contain polyribosomes, mitochondria and rough endoplasmic reticulum. Note the tight junctions (*TJ*) between the cells at the apical ends. 12,000 ×

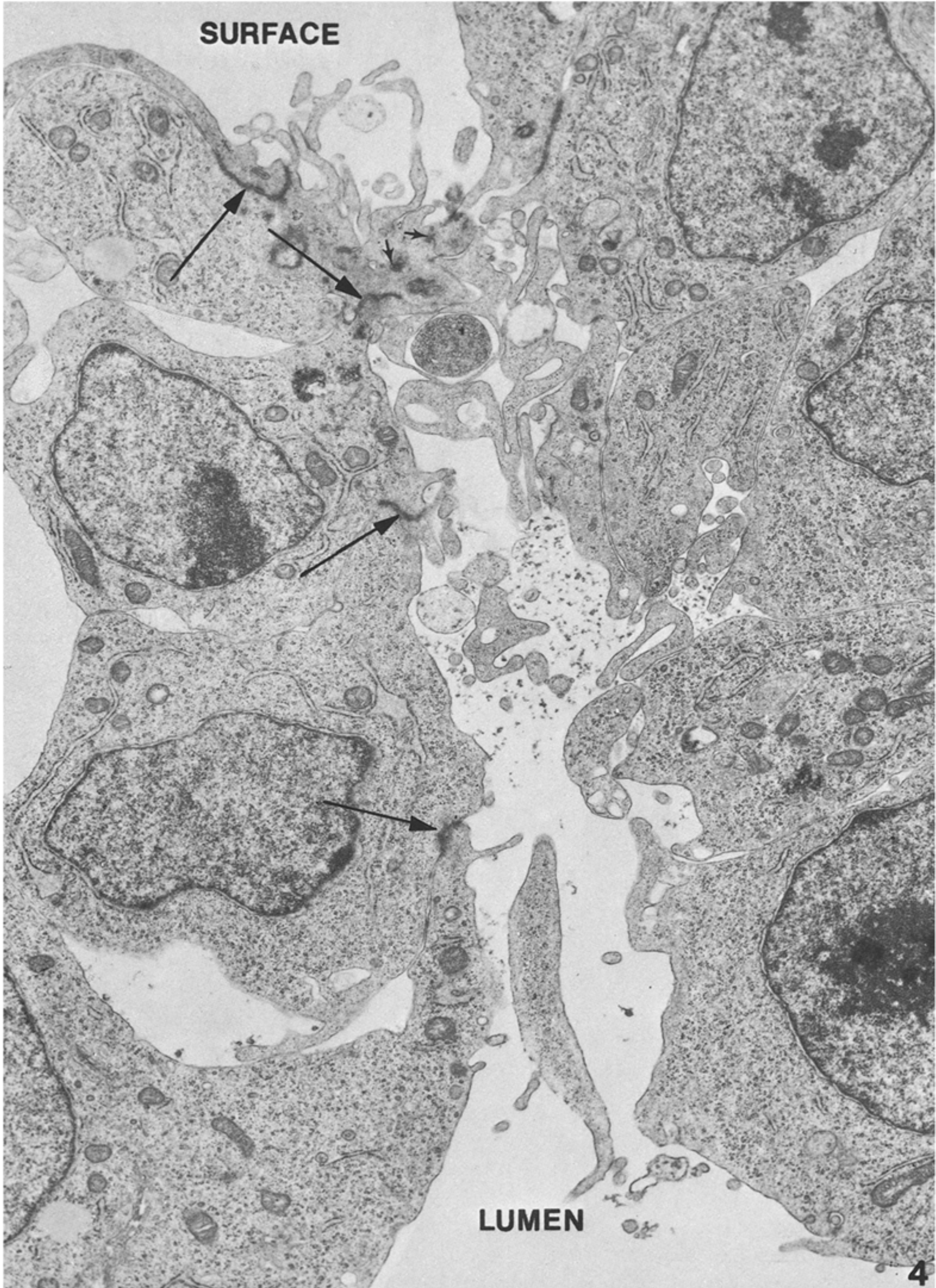


Fig. 4. Initial contact between the opposing rhombencephalic walls. Numerous extensions protrude in the gap between the two rims. They are particularly evident in the region close to the surface where previously the ruffles were seen. Note the small electron dense areas (arrowheads) between the projections of the two opposing walls. Also note the contact areas (arrows) between adjacent cells on the neural rim. 11,000 ×

the opposite wall. Some of these projections originated from surface ectoderm cells but others from cells located immediately below the surface. These latter cells did not resemble the attenuated flat epithelial cells seen before the closure, nor did they resemble the elongated neuroepithelial cells. They probably represent neural crest cells. Although distinct surface specializations were visible between adjacent cells in the neural wall (Figs. 3 and 4), in the contact area between the opposing walls only a few very small electron dense areas were observed (Fig. 4). Slightly further into the fusion area both the surface ectoderm cells and the cells immediately under the surface (possibly neural crest cells) established broad contact with similar cells from the opposite wall, but no elaborate junctions between opposing cells were seen (Fig. 5). Still further into the fusion area the neuroepithelial cells contacted each other and became aligned with their apical ends towards the lumen. Hence, the first contact in the rhombencephalic region was established by surface ectoderm; this was followed by contact between (possibly) neural crest cells and finally by contact between the neuroepithelial cells.

Closure of the Mesencephalon. On approaching the fusion area in the mesencephalon, the cellular composition of the rim of the neural wall changed. The flat attenuated epithelial cells were absent, and the surface ectoderm cells now extended over the tip of the rim to make direct contact with the neuroepithelial cells.

In the fusion area many extensions of the surface ectoderm interdigitated with similar structures from the opposite side, a picture greatly similar to that observed in the rhombencephalon. This initiale contact was soon followed by a stage characterized by more extensive and elaborate junctions than seen in other areas (Fig. 6). The precise nature of these junctions was difficult to determine but adjacent to them were usually found areas containing a dark filamentous material (Fig. 6). Hence, the initial contact in the mesencephalon was established by surface ectoderm cells and this contact seemed to be more elaborate than in other areas. Subsequently the neuroepithelial cells of the opposing walls made contact by large cytoplasmic extensions, but failed to develop any membrane specializations comparable to those seen between the surface ectoderm cells. Finally the neuroepithelial cells became oriented perpendicular to the lumen, and the regular membrane specializations developed at their apical ends (Fig. 3).

Closure of the Anterior Neuropore. In the region of the anterior neuropore the neural walls were initially separated by a relatively wide gap. The rims were covered by surface ectoderm, which was in direct contact with the neuroepithelial cells further down in the gap. Closer to the fusion area the surface ectoderm receded and the first contact between the opposing walls was established by the neuroepithelial cells which faced each other with their apical ends (Fig. 7). Numerous small processes protruded from the cells (Fig. 8). At their distal ends they contained some flocculent material, but with the exception of some ribosomes, other organelles were lacking. No distinct membrane specializations were seen between processes from cells of the opposing walls. Hence, although

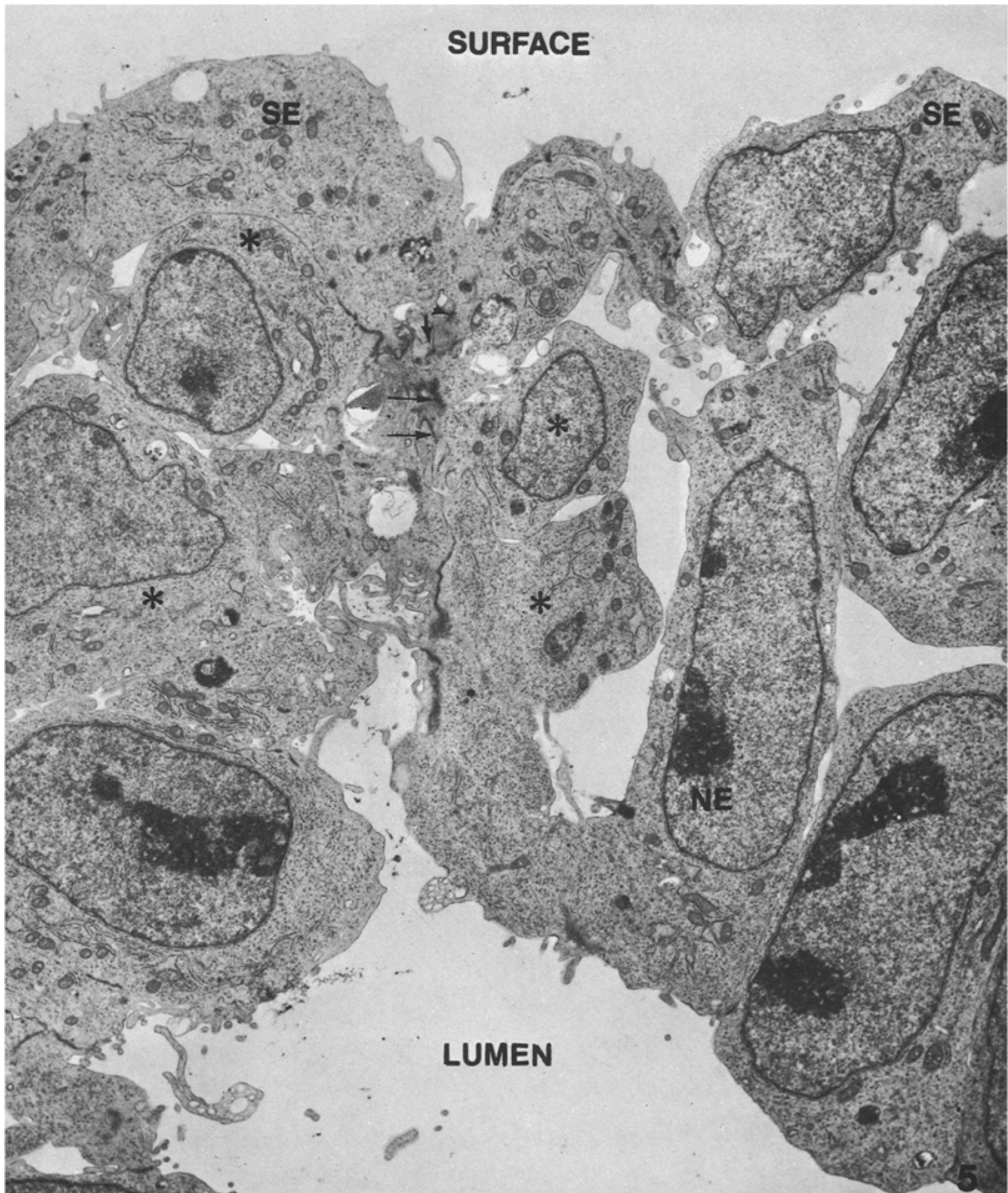


Fig. 5. Section slightly further into the area of fusion of the rhombencephalic walls as in Fig. 4. At the surface contact seems firmly established and a number of small electron dense areas (arrowheads) are visible. In the region just below the surface numerous cytoplasmic extensions occupy the space between the two walls and numerous small cytoplasmic extensions interdigitate with each other. Note also the neuroepithelial cells (*NE*). The cells immediately beneath the surface ectoderm probably represent neural crest cells (*). 5,000 ×

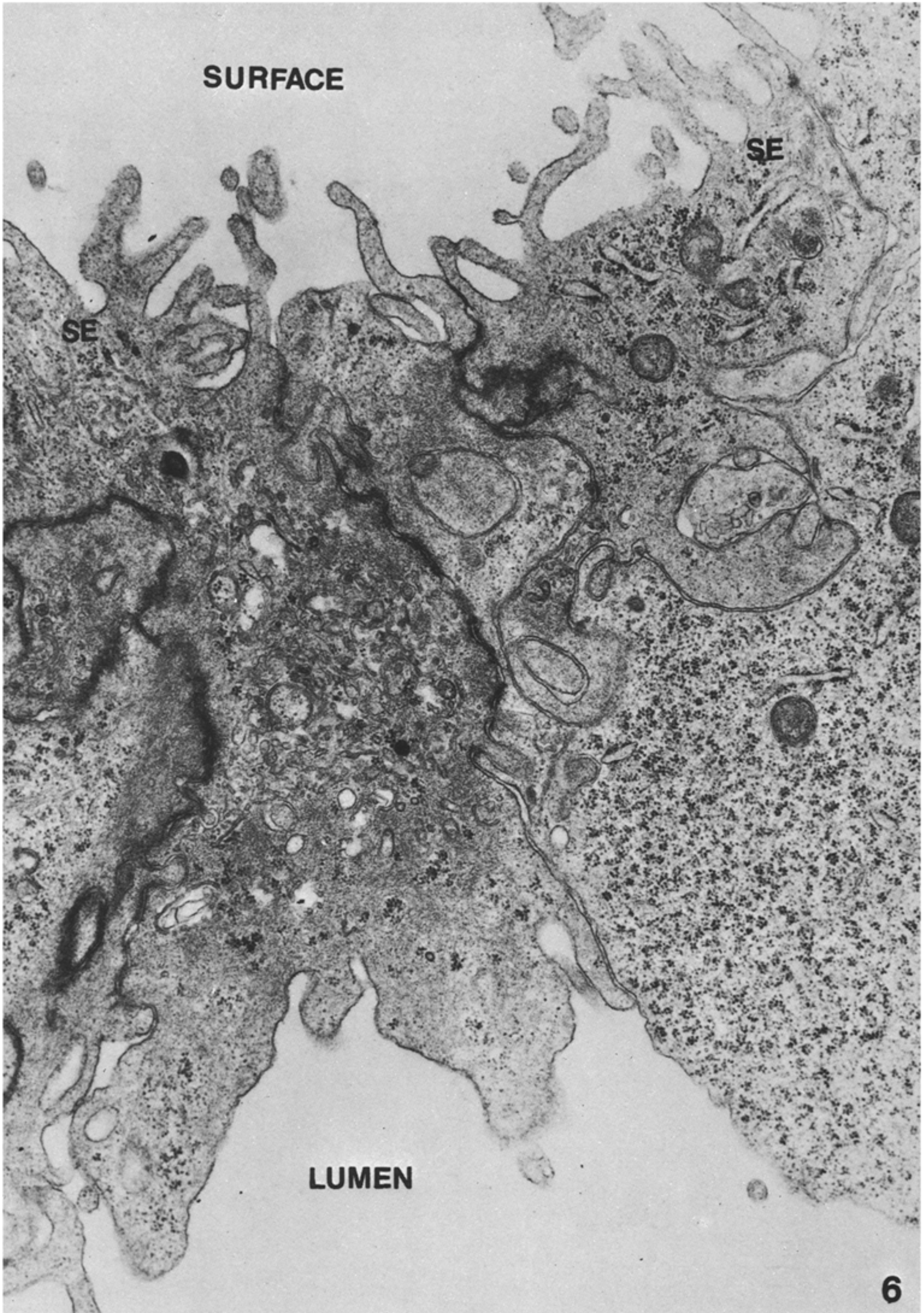


Fig. 6. Transverse section through the fused region of the mesencephalon. The surface ectoderm (*SE*) cells, characterized by numerous microvilli, have established firm contact between the two rims. In the fused area several electron dense junctional complexes extending over considerable distance are visible. Note the filamentous material beneath the junctions. 18,000 ×

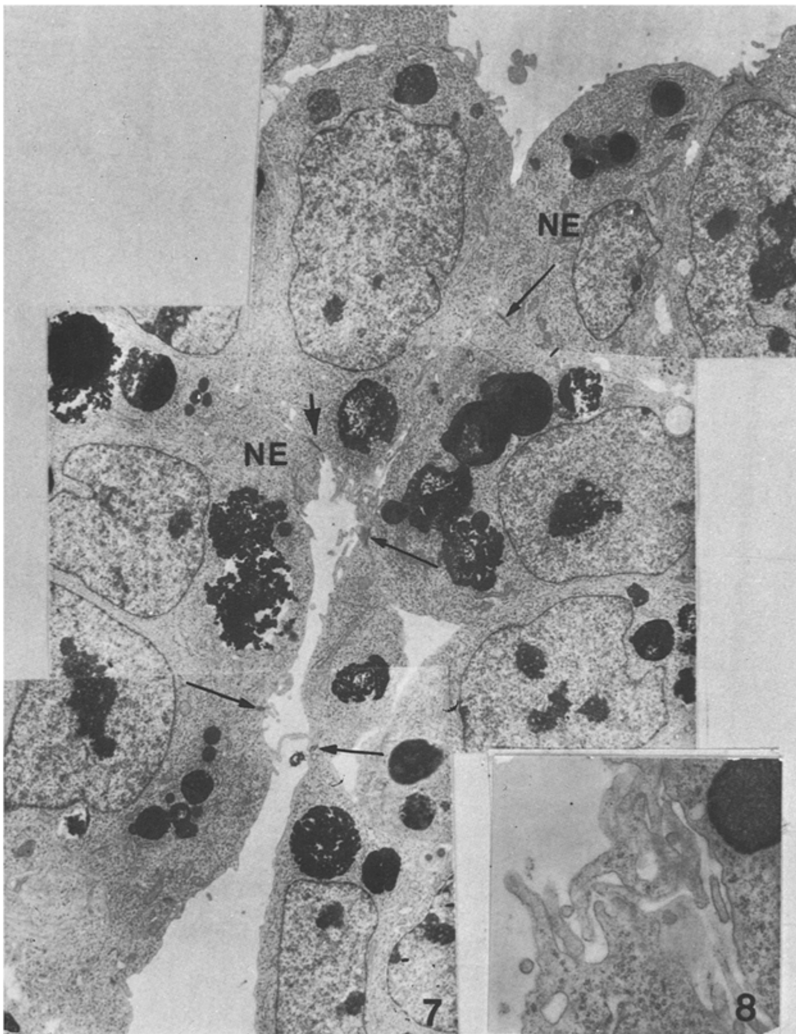
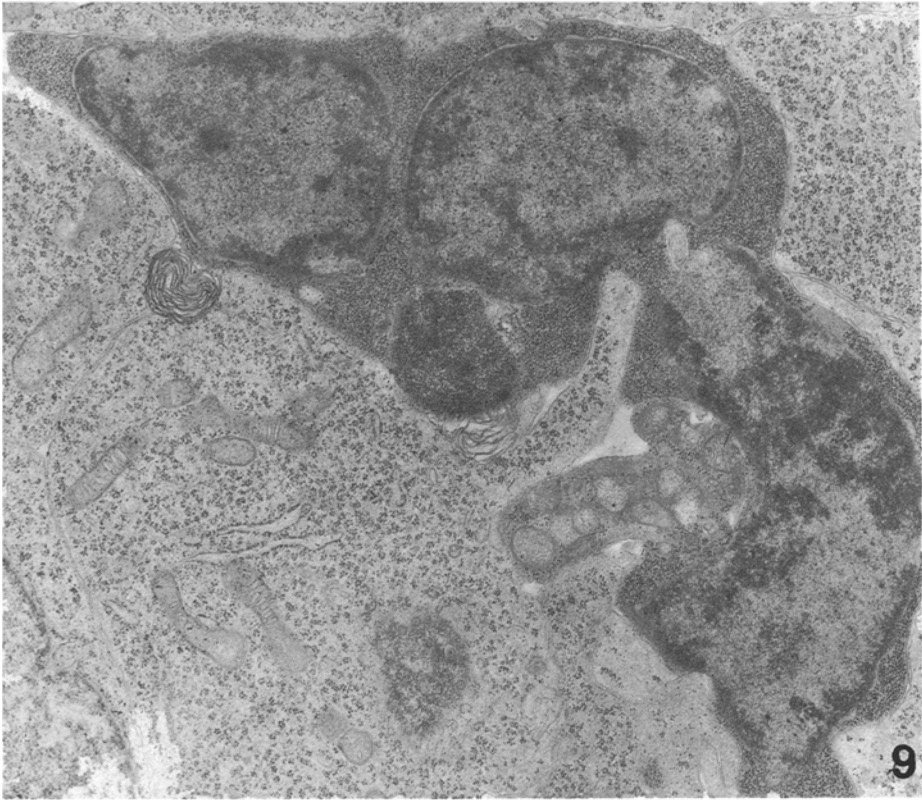


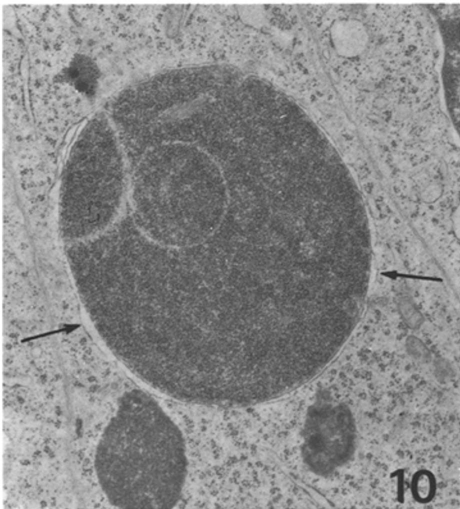
Fig. 7. Transverse section through the walls of the anterior neuropore showing the beginning of fusion between neuroepithelial cells (*NE*) of the opposing walls. Note the junctions (arrows) between adjacent neuroepithelial cells and the numerous cytoplasmic processes extending in the gap. Most neuroepithelial cells contain phagocytosed particles consisting of degenerated debris. Degenerating cells in the process of condensation and fragmentation are absent. 3,000 ×

Fig. 8. Magnification of cytoplasmic extensions arising from the apical ends of the neuroepithelial cells. They contain some flocculent material and ribosomes. 12,000 ×

distinct membrane specializations were found between adjacent neuroepithelial cells on either side of the groove, no such junctions were observed between the apical ends of opposing cells (Figs. 7 and 8).

In sections still further into the fusion area, the apical ends of the neuroepithelial cells gradually changed position from facing the gap between the two oppos-





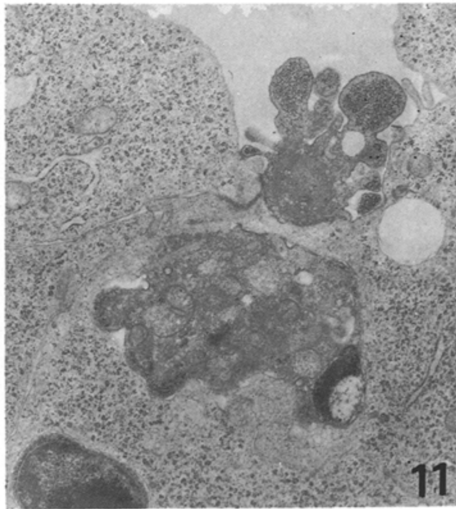


Fig. 9. Degenerating cell characterized by condensation of the nucleus and cytoplasm, the typical features of physiological cell degeneration. Note that a cytoplasmic arm of a healthy neuroepithelial cell begins to penetrate the dying cell. 12,500 ×

Fig. 10. Degenerating particle phagocytosed by healthy neighbouring neuroepithelial cell. Note the membranes around the phagocytosed particle. 8,000 ×

Fig. 11. Phagocytosed particles being extruded by the cell into the lumen. Some of the particles contain nuclear, others mainly cytoplasmic remnants. 6,400 ×

ing walls to facing the lumen of the newly formed tube. The impression was gained that the cells rotated about 90 degrees and that the apical ends from opposing cells were sliding along each other till they faced the lumen of the tube. Then a typical pseudostratified neuroepithelial pattern was established in the fusion area. The surface ectoderm covered the gap only after the neuroepithelial cells had established a pseudostratified epithelium.

Closure of the Prosencephalon. In this area surface ectoderm and neuroepithelial cells contacted each other approximately at the same time. Many thin cell processes extended from the surface ectoderm cells and established distinct junctional complexes similar to those seen in the mesencephalon (Fig. 6). The neuroepithelial cells made contact by multiple irregular processes originating from the apical aspects of the cells, but failed to form any membrane specializations. Similarly as in the region of the anterior neuropore the neuroepithelial cells soon after fusion formed a regular pseudostratified epithelium with the apical ends at the lumen of the tube where they were connected by junctional specializations.

Cell Degeneration. Cell death was observed in the rhombencephalic region before fusion occurred, but usually little cell debris remained when contact was established. In the anterior neuropore and prosencephalic regions, however, cell degeneration was seen before, during and after fusion (Fig. 7). Only a few cells showed condensation of the nuclear chromatin and cytoplasm, the typical characteristics of on-going physiological cell degeneration (Fig. 9). In many cases the degeneration had proceeded to fragmentation and many fragments were surrounded by cytoplasmic arms or completely enclosed by healthy surrounding neuroepithelial cells (Fig. 10). In some of the phagocytosed particles the original nuclear components and cytoplasmic organelles could easily be distinguished, but in others those features were lost and only amorphous material could be seen. With further degradation clear areas appeared in the phagocytosed cell debris (Fig. 7). Initially the phagocytosed particles were surrounded by their own membrane (Fig. 10), but later this membrane disappeared (Fig. 7). Not infrequently parts of dying cells were extruded into the lumen (Fig. 11). Specialized macrophages were not observed. Hence, during the fusion process few dying cells were observed, but in the region of the anterior neuropore and prosencephalon the neuroepithelial cells contained many darkly stained particles representing the remnants of phagocytosed degenerated cells. The neuroepithelial cells participating in the actual fusion process did not show signs of degeneration themselves.

Discussion

In recent years a number of ultrastructural studies have been performed on the fusion of opposing swellings and ridges in the embryo. In our own department the fusion of the medial and lateral nasal swellings and of the neural walls was examined with the ruthenium red technique and an opaque substance

was found over the free surface of the epithelial linings (Gaare and Langman, 1977; Sadler, 1978). This surface coat, probably glycoprotein in nature (Leblond and Bennett, 1974; Luft, 1976), was often thickest in the region of prospective contact and tapered off in nasal and oral directions. It was suggested that the coat may play an important role in cell recognition and in mediating the initial contact between processes from opposing cells. Greene and Kochhar (1974), Souchon (1975), Pratt and Greene (1975), and Pratt et al. (1975) similarly found a cell coat over the medial edges of the palatal shelves, the prospective contact regions in the palate. When Pratt et al. (1975) prevented the formation of the surface coat by treatment with diazo-oxo-norleucine (DON), the palatal shelves failed to adhere, indicating that the coat may be important in mediating adhesion between opposing palatal shelves. Examining the fusion of the neural walls Moran and Rice (1975) and Sadler (1978) also found a heavy surface coat on the rims of the neural folds in amphibia and mice, respectively. In the mouse embryo the coat was particularly heavy in the region of prospective contact. Hence, although in our ultrastructural studies we did not stain for the surface coat, it must be realized that in addition to the direct cellular contact between the cells, the surface coat may also be essential in the closure of the neural tube.

Permanent contact between cells of opposing nasal swellings was established by small projections arising from the surface of the cells (Gaare and Langman, 1977). Where the membranes touched, they were temporarily characterized by increased electron density, but distinct cell membrane specializations such as desmosomes were never observed. Fusion between the endocardial cushions of the chick heart was studied by Hay and Low (1972) and Los and van Eijndthoven (1973). The initial contact between the cushions was established by small and sometimes tongue-like processes. Specialized junctional complexes were never found with exception of a few small junctions in the region of apposition. In the contact region between the palatal shelves, Hayward (1969) found a few desmosomes between the contacting epithelial cells, but Farbman (1968) was unable to detect any membrane specializations between the shelves. Similarly Hinrichsen and Stevens (1974) failed to see any distinct membrane specializations between the shelves. Hence, few if any specialized junctional complexes are found in the fusion zone of the palatal shelves, nasal swellings, and endocardial cushions.

The fusion mechanism between the neural walls is different from that in the palate and lip. This is not surprising since the epithelial seam formed by the epithelial linings of the palatal shelves and nasal swellings disappears shortly after fusion has been established. Similarly the cells lining the endocardial cushions disappear shortly after contact has been established and are transformed into mesenchyme cells (Hay and Low, 1972). When the walls of the neural groove fuse, however, no epithelial or cellular seam is formed and little cell degeneration is seen, with exception of the anterior neuropore and prosencephalic regions. An additional difference between the fusion of the palatal shelves, nasal swellings, endocardial cushions, and the neural walls is the presence of electron dense membrane specializations between the surface ectoderm cells. This is understandable since the surface ectoderm forms a permanent bridge

between the two sides. This bridge will not disappear in contrast to the epithelial seam between the palatal shelves and nasal swellings.

Contact between the neuroepithelial cells of opposing walls in the prosencephalic and mesencephalic regions is initially established by numerous finger-like cellular projections which interdigitate with each other. These projections, however, fail to form any specialized junctional complexes. Since shortly after fusion, the neuroepithelial cells orientate themselves with their apical ends to the lumen, the formation of specialized junctional complexes would make this orientation very difficult. In all probability the cells "slide" along each other until the final position and orientation have been reached. It seems to us that the closing mechanism in the prosencephalon, the anterior neuropore region and the mesencephalon is principally the same, with the only difference that in the mesencephalic region the surface ectoderm cells make the first contact, while in the other two areas the initial contact is established by the neuroepithelial cells.

The fusion of the rhombencephalon was characterized by some special features. Initially the rhombencephalic wall was made up of surface ectoderm cells with numerous microvilli, neuroepithelial cells with apical protrusions, and a row of flat cells as a transition between the two. At the junction between the flat epithelial cell and the surface ectoderm cell were frequently seen large cytoplasmic extensions protruding into the gap between the two walls. These surface structures were more elaborate than those on the surface ectoderm or at the neuroepithelium and are probably comparable to the ruffles described and illustrated by Waterman (1976). When this investigator studied the closure of the neural groove with the scanning electron microscope, numerous lamellapodia or ruffles were observed to protrude between the presumptive surface ectoderm and the flat cells. It was frequently impossible to determine from which cell type they originated. Indeed, in our own study we sometimes gained the impression that the protrusions originated from the surface ectoderm cells and sometimes from the flat cells.

The contact between the two walls was not only made by the large ruffles but also by extensions from other cells. The nature of this latter type of cells was difficult to determine by morphological characteristics. They did not resemble surface ectoderm cells characterized by microvilli, nor neuroepithelial cells characterized by apical protrusions and sharply delineated tight junctions. In addition, the flat cells seen earlier could not be detected when actual fusion occurred. According to Waterman (1976) the flattened cells participate in the attachment between the neural folds and then lose their extensive contacts with both the surface and neuroepithelial cells to become neural crest cells. This suggestion raises the possibility that the initial contact in the rhombencephalon is established by neural crest cells. Indeed, Martin-Padilla (1970) also suggests that the neural folds in the hamster and mouse make their initial contact at the level of neural crest cells. Apparently the neural crest cells have the ability to form large cytoplasmic arms designed to establish points of adhesion with opposing similar cells (Harris, 1973; Revel, 1974). The neural crest cells of the opposing walls fail to form highly specialized cell junctions. This is understandable since they will migrate bilaterally to participate in the formation

of their respective tissues as soon as firm contact between the surface ectoderm cells has been established. Hence, it is highly likely that in the rhombencephalic area both the surface ectoderm and neural crest cells play an important role in the fusion between the walls.

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