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Neural Tube and Neural Crest: A New View With Time-Lapse High-Definition Photomicroscopy

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The dynamic process of neural tube formation and neural crest migration in live, unstained cultured avian embryos at Hamburger-Hamilton (H.H.) stages 8–11 was investigated by time-lapse cinematography using a high-definition microscope. These studies have demonstrated that neural tube closure in the trunk region differs from that observed in the head. The cephalic neural folds elevate slowly, then make contact rapidly. Following this initial apposition, they gradually "zip-up" in the rostrad and caudad direction. In the trunk region where the neuroepithelium bulges adjacent to the somites, the edges of the folds pulsate and forcefully touch-retract-touch in these bulging regions; the intersomitic epithelia retract, remain open even after more posterior somitic regions have apposed, and then close slowly. Epithelial blebs and N-CAM antibody were observed at the leading edges of the neuroepithelia. Between the open folds only a few bridging cells were seen; they probably represent the sites of initial cell adhesion following epithelial retraction. Focusing into the developing embryo shows that neuroepithelial fusion occurs prior to surface epithelial fusion. A meshwork of synchronously pulsating neural crest cells was identified below the surface epithelium and a preliminary investigation of their initial migration was conducted.

KEY WORDS: brain development, N-CAM, avian embryo

INTRODUCTION

The central nervous system, one of the first organs to develop, is critical for neonatal function and survival. Neural tube defects are among the most common in humans [Myrianthopoulos and Melnick, 1987], the incidence in the first trimester being 7–8-fold greater than at birth; most appear to be aborted early [Nishimura and Okamoto, 1976]. Although the pathogenesis of neural tube defects is not completely clear, it has been suggested that abnormalities such as anencephaly and spina bifida are most likely related to failure of the neural tube to close [see reviews, Lemire et al., 1978; Campbell et al., 1986; Lemire and Siebert, 1990].

Neurulation can be subdivided into three stages: 1) the thickening of the presumptive epithelium to form the neural plate, 2) neural fold elevation, and 3) neural tube formation resulting from the apposition and fusion of the dorsal edges of the neural folds. Each of these stages has been investigated extensively by light, scanning (SEM), and transmission (TEM) electron microscopy [Bellairs, 1959; Bancroft and Bellairs, 1975; Portch and Barson, 1974; Sakai, 1988; Waterman, 1975, 1976, 1979]. Recently, several studies have focused on the possible mechanisms of neural fold elevation and fusion [Nagele and Lee, 1987; Nagele et al., 1987, 1989; Schoenwolf 1985; Schoenwolf and Franks, 1984]. Yet despite this extensive research, several outstanding questions concerning neural tube formation remain unanswered. For example, still unclear is the exact nature of neural fold movement, and whether the early brain and spinal cord form by a similar mechanism.

To begin to address these and other developmental questions, we have investigated chick neurulation by time-lapse cinematography using a high-definition light microscope. This technique permitted a close view of living, unstained embryos during the dynamic process of neural tube formation. We have demonstrated that neural tube closure in the cephalic region differs from that observed in the trunk. Throughout the forming neural tube, extensive cellular activity was seen within and between neural folds. On the leading edges of the apposing neuroepithelia, epithelial blebs and cell adhesion molecules (N-CAM) were observed. A meshwork of pulsating neural crest cells were identified be-

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low the epithelial surface and a preliminary investigation of their initial migration was conducted.

MATERIALS AND METHODS

Chick Embryos

White leghorn eggs *(Gallus gallus domesticus)*, obtained from K and R (Westminster, CA), were incubated in an humidified, air-forced incubator at 37°C for 32–45 hours. A small 5 × 3 mm opening in the egg shell was made and the embryos were vital-stained with a 1% solution of neutral red. The chicks were staged according to Hamburger-Hamilton (H.H.)(1951) and viewed under a dissecting or high definition light microscope. Thirty vital-stained embryos were observed in ovo.

In Vitro Technique

Eggs were incubated as described above and the embryos were carefully cut off the yolk, washed twice in warm phosphate buffered saline (PBS) and three times in warm BGJ_b-enriched culture medium (Gibco, Grand Island, NY) containing 10 mM HEPES buffer. Stage 8–1 embryos were placed in a 35 mm plastic Petri dish and overlain with a 1 cm diameter stainless steel ring. Each ring was filled with BGJ_b medium and covered with a 20 cm diameter round glass coverslip. The embryos were maintained at 37°C for 1–2 hours and examined under the high-definition light microscope. More than 40 embryos per stage were examined.

Histology

Following in vitro examination, selected embryos were fixed, stained, and examined. For comparison, 5 in ovo embryos from H.H. stages 8–11 were dissected from the yolk and immediately fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer for 1.5 hours at room temperature. The tissue was washed with buffer three times (30 minutes each wash), dehydrated in graded alcohols, cleared in histoclear, and embedded in paraplast. The embryos were sectioned at 3 μm in a plane perpendicular to the neural (long) axis. The sections were stained with hematoxylin and eosin and examined under a compound microscope. Cultured embryos exhibited morphology and histological structures identical to embryos developing in ovo at corresponding stages (data not shown).

Immunohistochemistry

H.H. stages 10–11 embryos were fixed in 99:1 ethanol-glacial acetic acid overnight at 4°C. The next morning, the embryos were washed in cold alcohol for 1 hour, cleared in cold histoclear for 1 hour, and embedded in paraplast. Embryos were serially sectioned at 3 μm in a plane perpendicular to the neural axis, mounted on gelatin subbed slides, and stored at 4°C for brief periods. The slides were rapidly deparaffinized in xylene, rehydrated in graded alcohol, rinsed in distilled water, and placed in PBS. The sections were incubated with monoclonal HNK-1 antibody (Becton Dickinson, Mountain View, CA) for 36 hours at 4°C, washed for 1 hr in PBS, and stained with biotinylated anti-mouse IgM (Vector, Burlington, CA)(1:10) for 45 minutes. The tissue was then washed twice in PBS (15 min each), incubated in FITC-conjugated streptavidin (Zymed, South San Francisco, CA)(1:2) for 30 minutes, and washed twice in PBS. The sections were mounted with aqueous media and stored at 4°C. Control slides included 1) biotinylated anti-mouse IgM and FITC-labelled streptavidin; and 2) HNK-1 followed by FITC-labelled streptavidin. Control sections were routinely negative for fluorescence.

For the whole mounts, embryos were fixed and stained following a modified method of Loring and Erickson [1987]. Briefly, embryos were fixed in 4% paraformaldehyde for 20 minutes, rinsed several times in PBS, and incubated in 3% bovine serum albumen (BSA) in PBS for 3 hours. All incubations were conducted at room temperature with gentle agitation. While in BSA, the ectoderm and/or endoderm was carefully slit to facilitate the penetration of the antibodies. The embryos were then incubated overnight with primary antibody, washed 30 minutes in PBS, and incubated with fluorescein-conjugated (FITC) secondary antibody for 3 hours. To identify neural crest cells, monoclonal HNK-1 antibody (1:5 dilution in PBS) followed by FITC-labelled anti-mouse IgM (Organon Teknika, Durham, NC, 1:50 in PBS) was used. To investigate the distribution of N-CAM during development, monospecific polyclonal rabbit anti-chick N-CAM (1:10 in PBS) was followed by FITC-labelled goat anti-rabbit IgG (Organon Teknika, Durham, NC; 1:75 in PBS). Secondary antibody alone was used as controls. The embryos were then washed in 0.5% BSA for 20 minutes postfixed in 4% paraformaldehyde for 10 minutes, washed 4 times in PBS, and mounted in depression chambers using 70% glycerol containing 0.1 $NaHCO_3$, pH 7.8, and 2% n-propyl gallate. Control sections were routinely negative for fluorescence.

Microscopic Evaluation

Embryos were examined with the Olbrich 4000 Light Microscope (Bayross, Ltd.). The Olbrich 4000 is a prototype microscope designed by Kurt Olbrich in West Germany. This instrument has a unique type of transmitted oblique illumination that can provide high definition images of thick, unstained preparations such as whole embryos or organ cultures. In this investigation, we utilized a high N.A. condenser with long working distance objectives (×10, ×20, ×40). Time lapse studies were conducted using a Bolex 16 mm movie camera. Film speeds were 60 FPM, 15 FPM, and 8 FPM, corresponding to 25×, 100×, and 200× normal speed.

The immunohistochemistry was observed using a Nikon epifluorescent attachment. The microscope is equipped with a high-pressure mercury burner and tungsten lamp (100 W). Photomicrographs were taken using Kodak Ektachrome 400 and TMAX 400 films.

Three-Dimensional Reconstruction

Three micron thick serial sections were photographed and prints of these sections were aligned on the Z axis. Using the PC-3D Program (Jandel Scientific, Corte Madera, CA) and a computer tablet, the neural tube, neural crest cells and surface epithelium were traced. A three-dimensional image was generated and transformed. These images were photographed using Ektachrome 100.

RESULTS

The morphology of the developing chick embryos was observed during neurulation by light microscopy and time-lapse cinematography using a high-definition light microscope; observations were identical for embryos either in culture or in ovo. In the cranial region of the early H.H. stage 8 embryo, the neural folds in the mesencephalic region elevated slowly and approached one other (Fig. 1). Once the folds were close to one other (Fig. 1), contact occurred relatively rapidly. The neuroepithelia did not approximate each other precisely; rather, one fold overlapped the second. The neuroepithelial cells were extremely active: pulsating, exchanging vesicles (Fig. 2) and moving within each fold. Following this initial epithelial apposition, cephalic closure progressed both anteriorly and posteriorly in a modified zipper-like manner similar to that previously described [Bancroft and Bellairs, 1975]. Neuroepithelial closure was not exact; small epithelial regions remained open, while adjacent anterior and posterior areas were apposed (Fig. 1D). All of these open areas subsequently closed with time (Figs. 3, 4). By H.H. stage 9, the cranial neural folds were apposed with only the anterior neuropore and posterior rhombencephalic neuropore remaining open (Fig. 4). Fusion had not yet occurred as indicated by the apposition line (Fig. 4).

In the trunk, the process of neural tube closure differed from that observed in the head. Posterior to the rhombencephalic neuropore, the epithelia bulged adjacent to the somites (Figs. 4–6). Within each neural fold numerous vesicles and blebs were observed in the leading edge epithelia (Fig. 6). The fold edges pulsated, approached each other relatively rapidly, and forcefully touched-retracted-touched at the bulging somatic regions (Figs. 5, 6). Frequently, one fold overlapped the second (Fig. 6B). In the adjacent intersomitic regions the neuroepithelia retracted and remained open even after more posterior somitic regions had already apposed (Figs. 5, 6); these areas then slowly closed (Fig. 7). The process of intersomitic apposition was much more gradual than that observed adjacent to the somites. Forceful collisions between the opposing folds were never seen in the intersomitic regions; the epithelia appeared to "zip-up" gradually. In addition, the rhombencephalic neuropore remained opened after more posterior regions had already closed (Fig. 7). The neuroepithelial cells were extremely active; the observed activity included pulsation, vesicular movement, and cellular movements within each neural fold and, less frequently, across the midline between folds.

To begin to characterize the sites of initial cell adhesion in the opposing neural folds, H.H. stages 9–10 embryos were stained with N-CAM antibodies. The distribution of N-CAM antigens is shown in Figure 8. On the leading edge of the open tube, a greater concentration of fluorescent stain was observed (Fig. 8); N-CAM was detected at a lower level throughout the neural epithelium (data not shown).

The closure of the posterior neural tube adjacent to nonsegmented mesoderm was observed during H.H. stages 9+ and 10 (8–10 somites). The process of closure

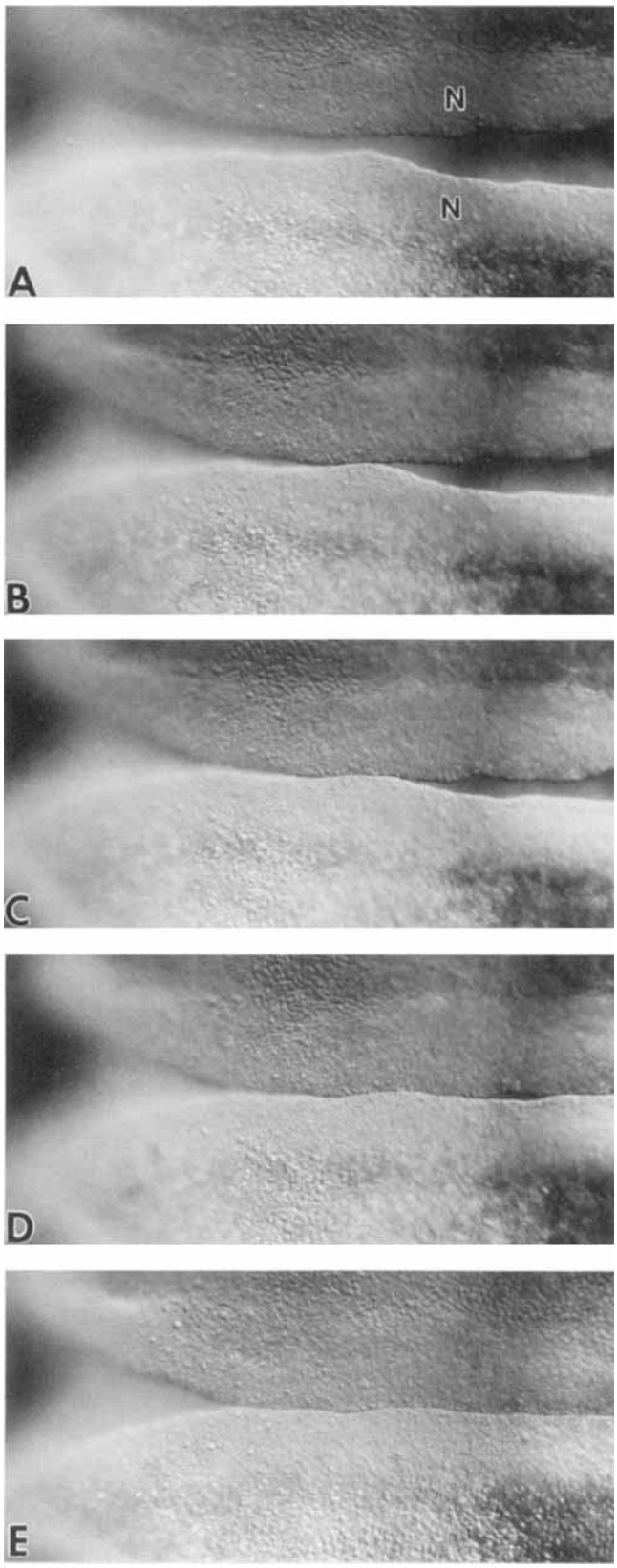

Fig. 1. Views of an early H.H. stage 8 embryo showing the sequence (**A–E**) of initial neural fold (N) apposition in the mesencephalic region. Anterior is to the left. ×160.

Fig. 2. A high-power view of neural fold apposition in the cranial region of a H.H. stage 8 embryo. Vesicles (arrows) are observed in the neuroepithelia. Small extracellular granules can also be observed moving in both directions along the axis of fold apposition when viewed in time-lapse. Anterior is to the right. ×640.

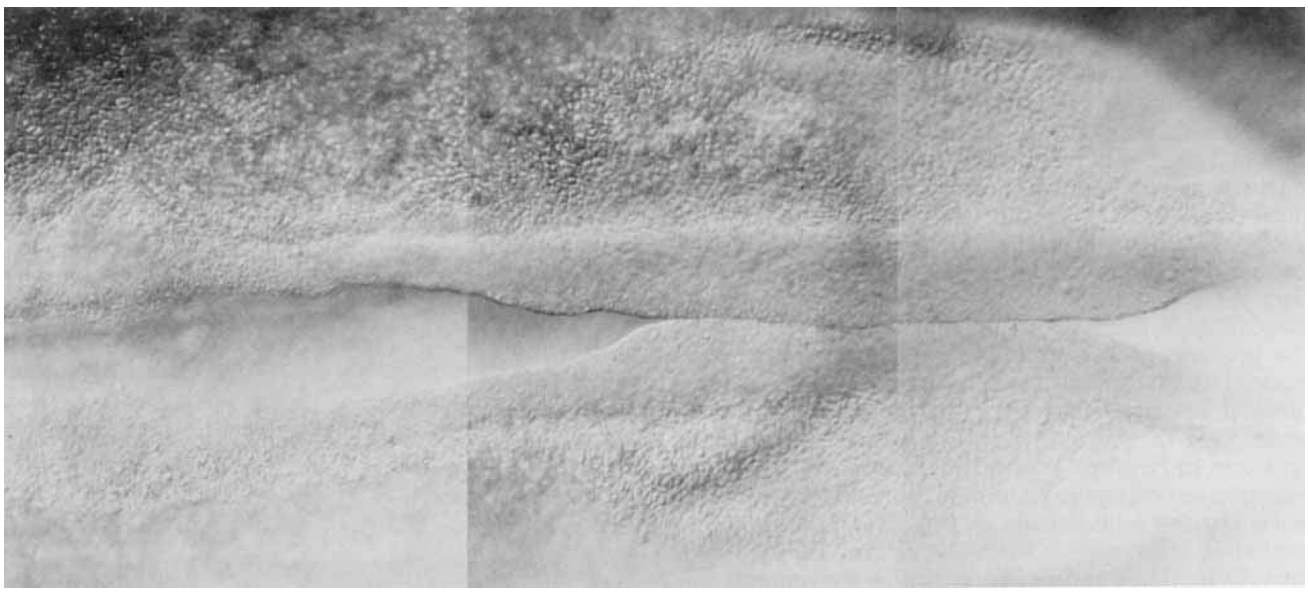

Fig. 3. Fold apposition in the cranial region is seen in a late H.H. stage 8 embryo. Fold apposition has progressed anteriorly (**right**) and posteriorly (**left**) from the site of initial contact. ×320.

Fig. 4. A low-power view of an early H.H. stage 9 embryo showing regionalization of the neural tube. The neural tube, closed in the cephalic region, has remained open at the anterior neuropore (arrowheads) and in the trunk region. In the trunk, neuroepithelial bulges are observed adjacent to the somites (s). Apposition line: arrows. ×160.

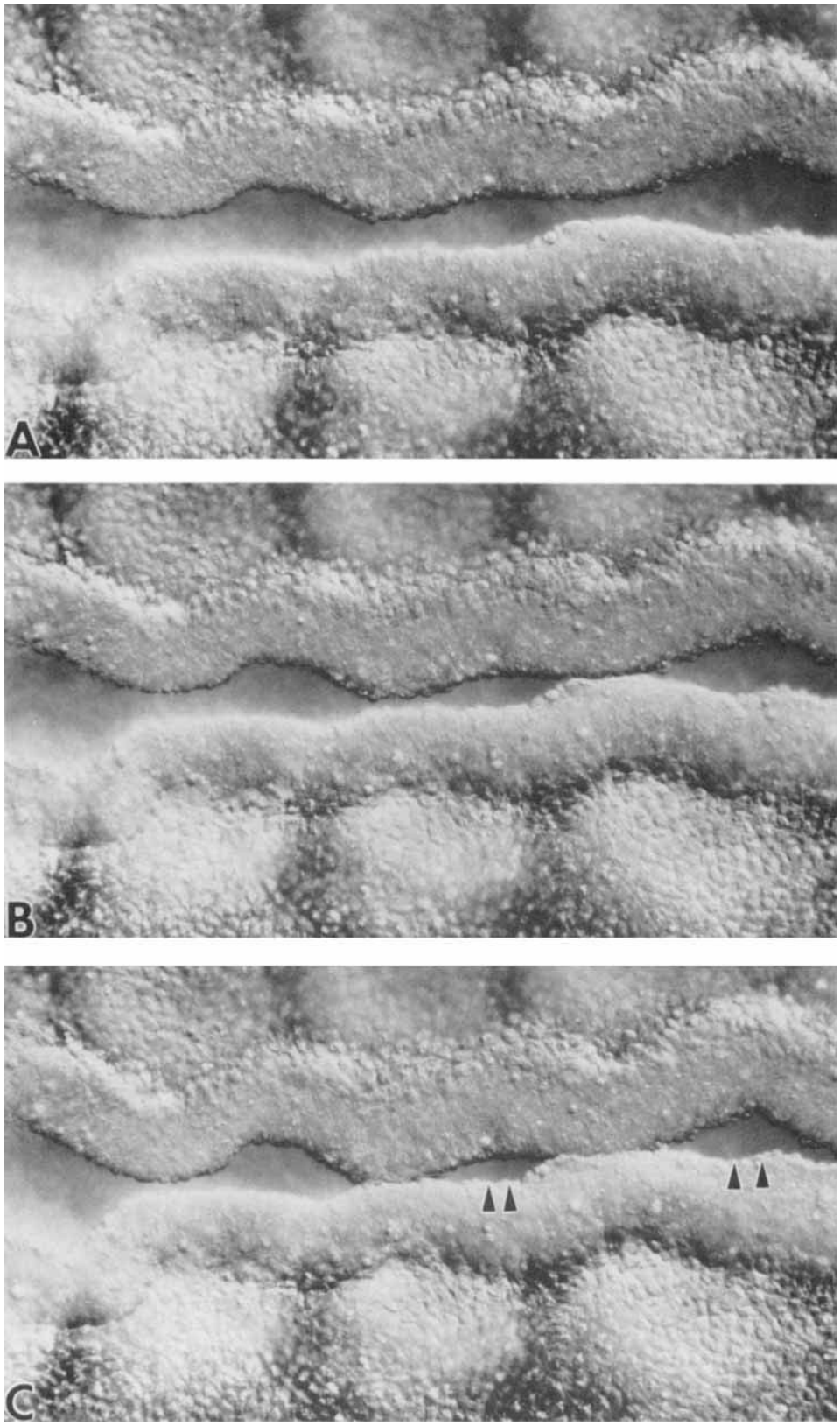

Fig. 5. A view of the sequence (**A–C**) of neural fold apposition in the trunk region of a H.H. stage 9 embryo. Initial contact was observed at the neuroepithelial bulges whereas in the intersomitic regions, the neural folds have retracted and remained open (arrowheads). ×320.

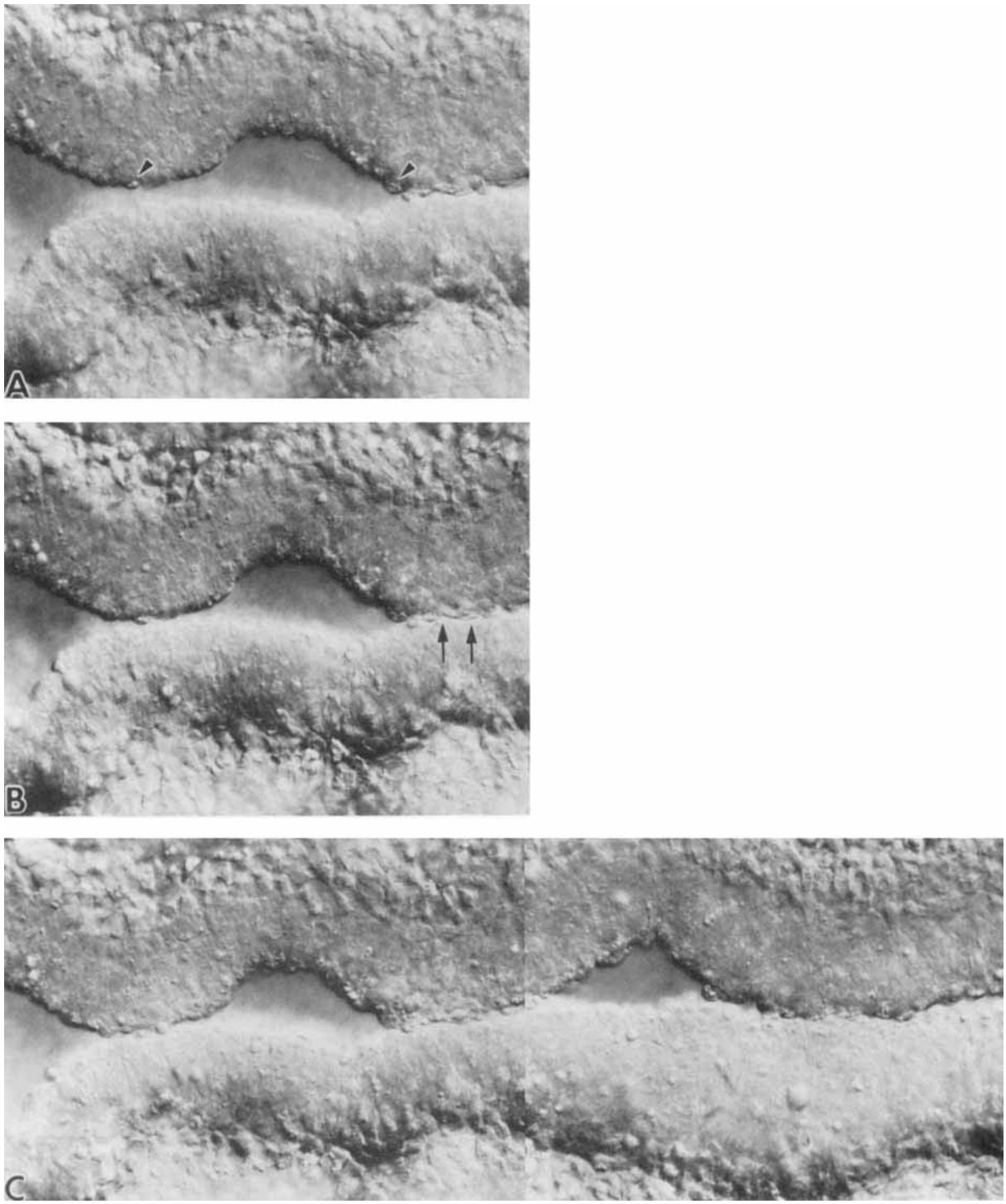

Fig. 6. A high-power view of the sequence (**A–C**) of neural fold apposition in the embryo shown in Figure 5 reveals cellular blebs (arrowheads) and associated granules at the leading edge of the apposing neuroepithelia. Frequently one fold overlaps the other (arrows). ×640.

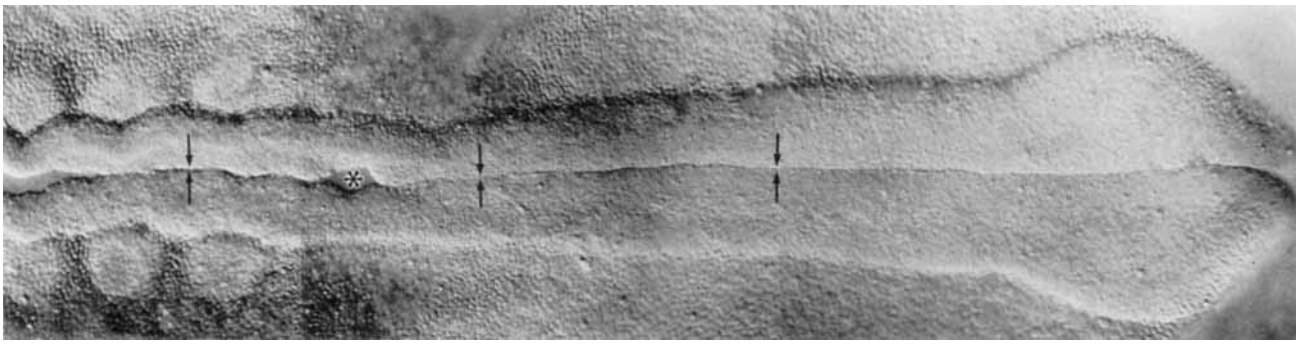

Fig. 7. A low-power view of a late H.H. stage 9 embryo. The rhombencephalic neuropore (*) has remained open after more posterior regions have already apposed; also note the lengthening apposition line (arrows). ×160.

closely resembled that exhibited in the cephalic region. Caudal to the 10th somite and in the absence of identifiable somites (Fig. 9), the neural folds gradually approached each other, and then apposed. Following occasional occurrences of neuroepithelia retraction after initial apposition, a few bridging cells were detected at the sites of initial contact (Fig. 9). These cells processes probably represent epithelial cells which adhered to the opposing epithelial surface and were pulled during the retraction process. Bridging cells were also occasionally demonstrated in the cephalic neuroepithelium (data not shown).

Neural Tube Fusion

With the magnifications, resolution, and lighting techniques being employed, we have defined fusion at the light microscopic level as the disappearance of the apposition line; the absence of a detectable line implies that the neuroepithelial cells are sufficiently close to each other such that they are attached by specialized cell-cell junctions. By H.H. stage 10, fusion was seen at the site of initial apposition (the mesencephalic region)(Fig. 10), even though the anterior and posterior neuropores still remained open. Fusion progressed both anteriorly and posteriorly in a pattern similar to that described above. Frequently, small isolated unfused areas were observed, which subsequently fused with time. Even at this relatively late stage of neurulation, extensive epithelial activity continued to be exhibited, including cellular and vesicular movements from one region to another.

To analyze the sequence of epithelial fusion (e.g.,

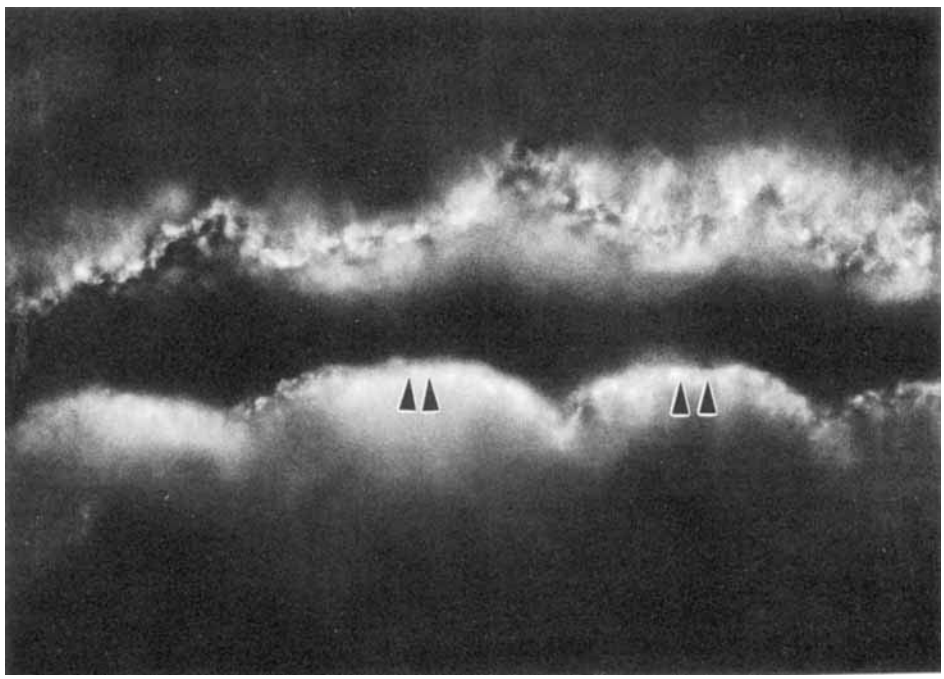

Fig. 8. Indirect immunofluorescent whole mount of a H.H. stage 9 embryo stained with N-CAM antibodies. A higher concentration of N-CAM antigens is observed (arrowheads) at the leading edge of the open neural tube. ×290.

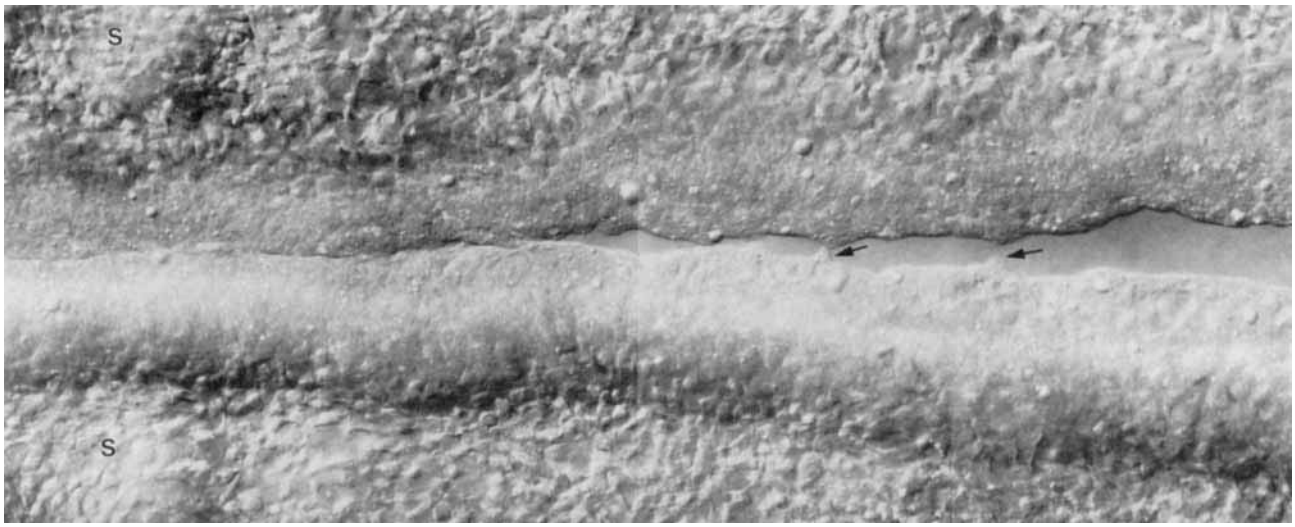

Fig. 9. A high-power view of fold apposition in the region posterior to the somites (s) in a H.H. stage 10 embryo. A few bridging cells (arrows) are observed coursing between the neural folds. Anterior is to the left. ×640.

whether fusion occurred from the dorsal surface downward or from the ventral surface upward), we focused into the living embryo. This enabled us to observe the neural (Fig. 11A) and surface (Fig. 11B) epithelia in unfixed embryonic tissue during the fusion process. Focusing into the embryo showed that neuroepithelial fusion occurred prior to the fusion of the surface epithelium.

Neural Crest Cells

Neural crest cells migrate from the neural folds throughout the developing chick in distinct pathways [see reviews Noden, 1988; Bronner-Fraser, 1987]. Using HNK-1 antibody and immunohistochemistry, we have identified the neural crest cell population in the cephalic region (Fig. 12); crest cells were localized in the cell free space between the surface epithelium and the neural tube (Fig. 12A). The distribution of the crest cell population was also determined by the whole mount staining technique (Fig. 12B) and 3-D reconstruction (Figs. 12C, D) of the developing cephalic region. The crest cells were observed anterioventral and posterioventral to the optic lobes and, in the mesencephalic region, lateral to the neural tube (Fig. 12B–D). Using high-definition time-lapse photomicroscopy, we were able to begin investigating initial crest cell migration in situ. A meshwork of

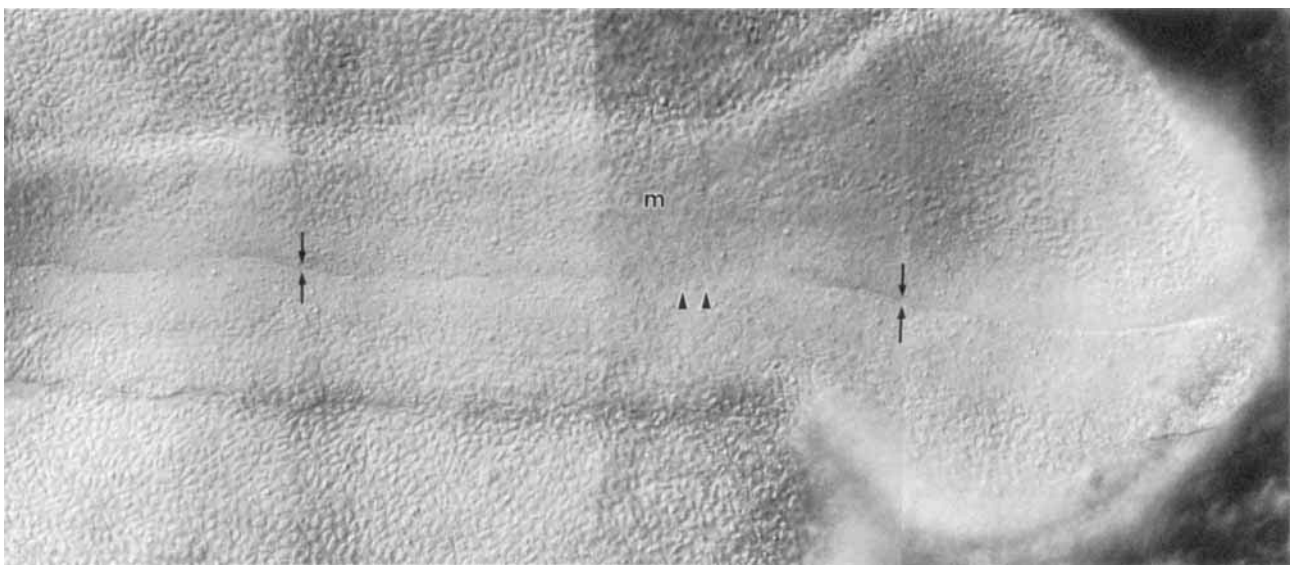

Fig. 10. Epithelial fusion in a H.H. stage 10 embryo. Fusion (arrowheads) has occurred in the mesencephalic region (m), the site of initial apposition. The remainder of the neural tube is apposed and unfused, as indicated by the distinct apposition line (arrows). ×320.

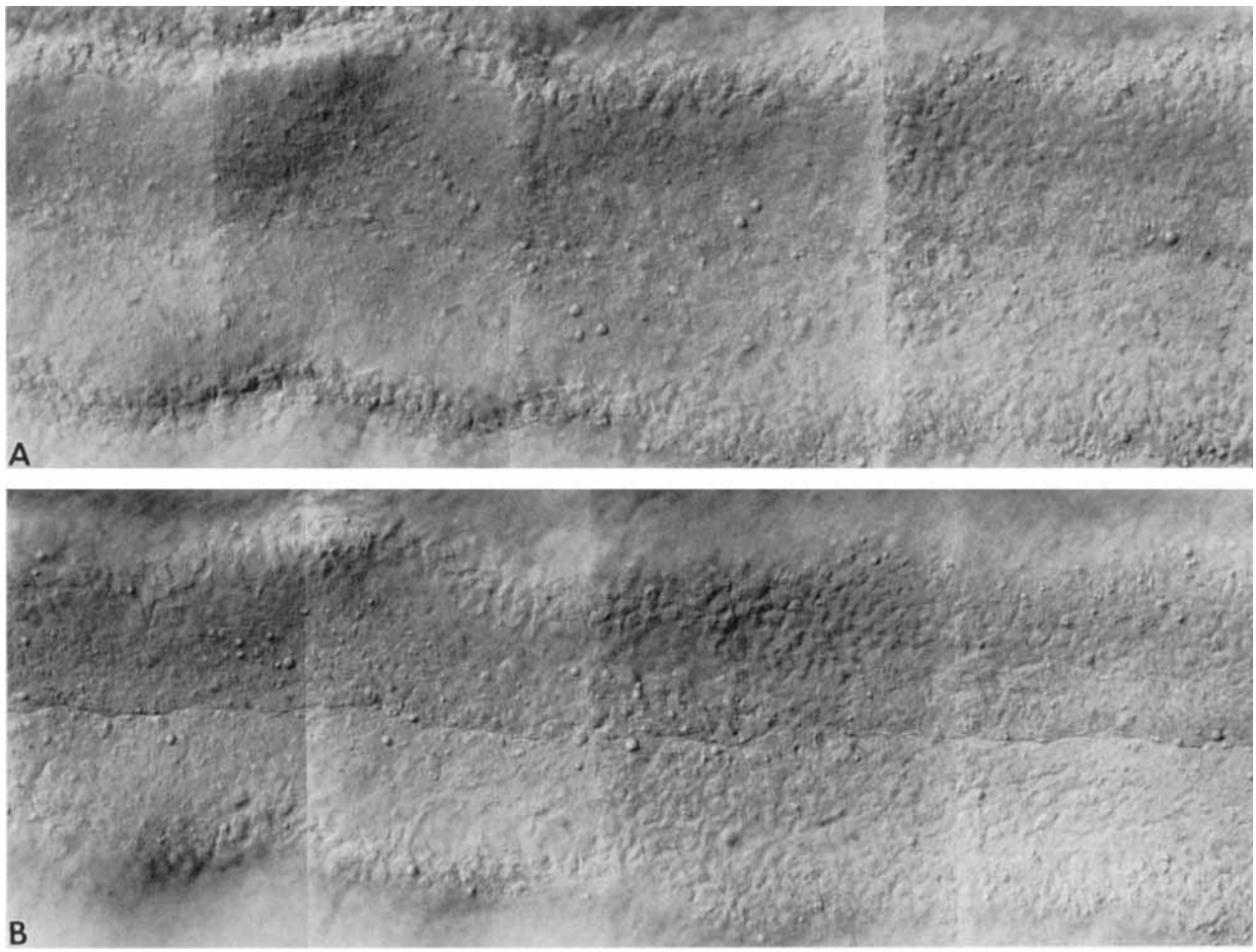

Fig. 11. Focusing into a H.H. stage 10 embryo demonstrates that the neural epithelium has fused prior to the surface epithelium. **A:** Neuroepithelium. **B:** Surface epithelium. ×320.

synchronously pulsating crest cells was seen below the surface epithelium lateral to the neural tube (Fig. 13). Occasionally, 1–3 individual cells lost their filopodial connections and migrated away from the field; we never observed large migrating crest cell populations.

DISCUSSION

Neurulation has been investigated extensively in birds [Bellairs, 1959; Bancroft and Bellairs, 1975; Portch and Barson, 1974], in mammals [Waterman, 1976, 1979; Sakai, 1988], and in other species [Tarin, 1971] by light and electron microscopy. In most of these studies, the delicate embryos have been exposed to fixatives and processing techniques prior to evaluation, increasing the risk of artifacts. In addition, the investigation of living embryos under relatively low magnification [Nagele et al., 1987] was unable to reveal the dynamic nature of neurulation. The recent availability of the high-definition light microscope has enabled us to observe the dynamic process of neural tube formation in living avian embryos at higher magnifications (×320–×640) and with better clarity than was previously possible. This microscope, the Olbrich 4000, coupled with time-lapse cinematography, provided an excellent close view of the developing neural tube.

We investigated neurulation in the avian embryo, H.H. stages 8–11. The process of fold apposition in the head region was significantly different from that observed in the trunk (compare Figs. 1–3 to Figs. 5–7). In the cephalic region, neural fold elevation occurred slowly and was quickly followed by forceful neuroepithelial collision and apposition. After the initial fold contact in the mesencephalic region (Fig. 1), tube closure progressed anteriorly and posteriorly in a manner similar to that reported previously [Bancroft and Bellairs, 1975]. Apposition occurred as an imperfect "zipping-up" in which small localized regions remained open while adjacent epithelia were already touching (Fig. 1D); with development, these open areas closed (Figs. 3, 4).

Observations in the region posterior to the rhombencephalon, the presumptive cervical region, showed a different closure pattern. Neural apposition occurred first at the bulging neuroepithelium adjacent to the somites (Figs. 5, 6). Although these neuroepithelial bulges had been reported in 1974 in a brief abstract [Gouda, 1974]

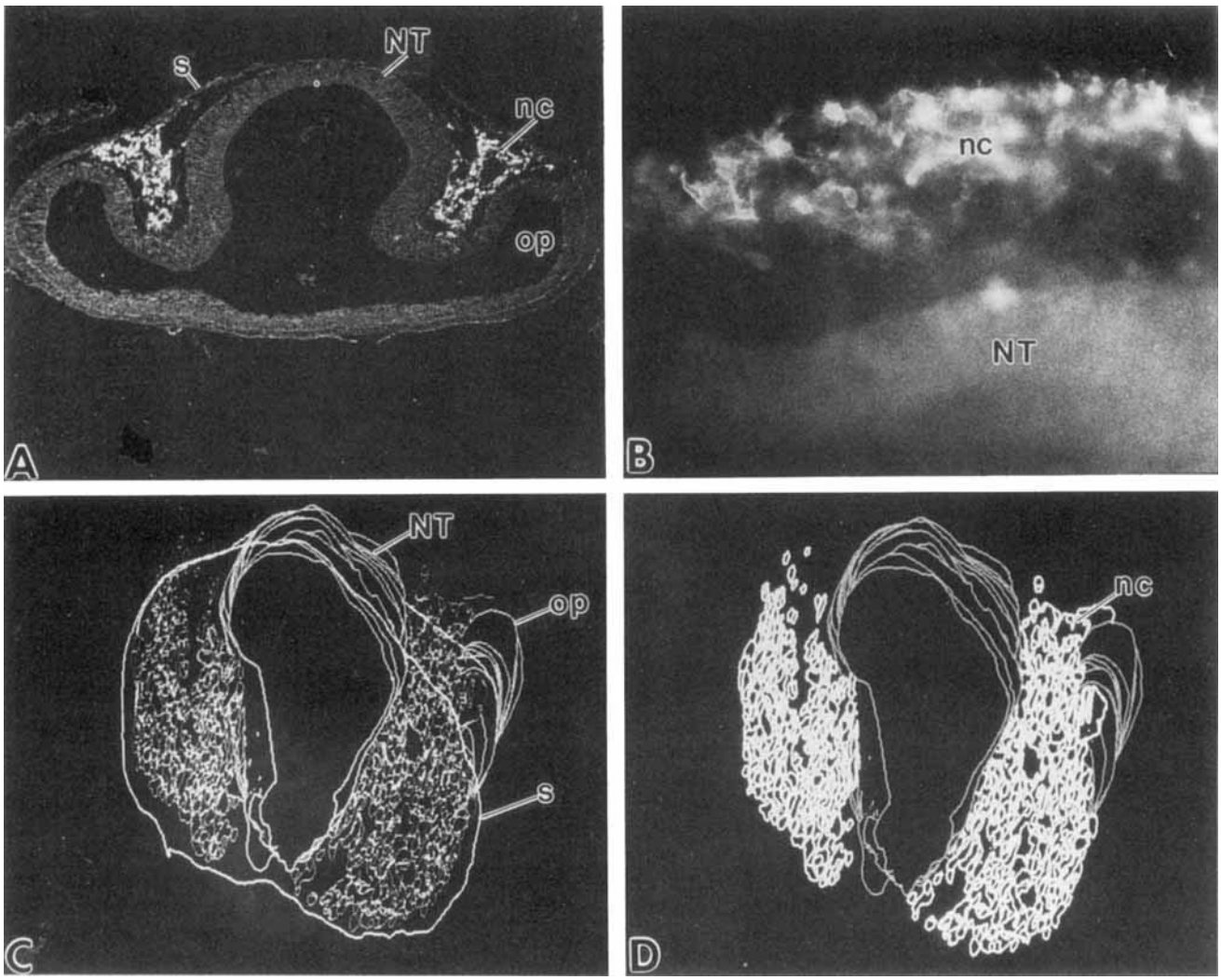

Fig. 12. The identification and distribution of cranial neural crest cells (nc) lateral to the neural tube (NT). **A:** Diencephalic region of a H.H. stage 11 embryo stained with HNK-1 antibody. ×125. **B:** Whole mount of a H.H. stage 10 embryo stained with HNK-1 antibody. ×230. **C, D:** 3-D reconstruction of a H.H. stage 11 embryo, viewed anteriorly from the mesencephalon, showing the distribution of the neural crest cells. Note the relationship of neural crest cells relative to the neural tube, the developing optic lobes (op), and surface epithelia (s).

and have been seen in other studies [Nagele and Lee, 1987, Fig. 1; Nagele et al., 1989, Fig. 3], this morphological pattern has been largely ignored. The apparent disinterest in these epithelial bulges was probably due to the lack of understanding of their developmental significance during neurulation. Time-lapse cinematography has demonstrated that initial fold apposition occurred at these neuroepithelial bulges, whereas the intersomitic epithelia retracted, remained opened, and then closed slowly (Figs. 5, 6). Regional differences in the "mechanisms" of tube apposition and the relative late closure of the rhombencephalic neuropore (Figs. 4, 7) may be associated with the relatively high incidence of neural tube defects in specific regions of the developing central nervous system, and low incidence in others. This possibility merits further investigation.

During the transformation of the neuroepithelium from a neural plate to a closed neural tube, epithelial morphology and cell surface coat material have been shown to change [Bancroft and Bellairs, 1975; Freeman, 1972; Lee et al., 1978; Sadler, 1978; Smits-van Prooije et al., 1986; Takahashi and Howes, 1986; Takahasi, 1988]. Evaluation by light and electron microscopy has demonstrated the presence of extensive epithelial folding, microvilli, and large blebs in the apical surface neuroepithelia [Portch and Barson, 1974; Bancroft and Bellairs, 1975]. A large number of bridging cells, with their filopodia extending between the opposing folds, were also observed [Waterman, 1976, 1979]. The presence of filopodia and microvilli extending between apposing folds has led to the hypothesis that they may play an active role in drawing the tube together [Waterman,

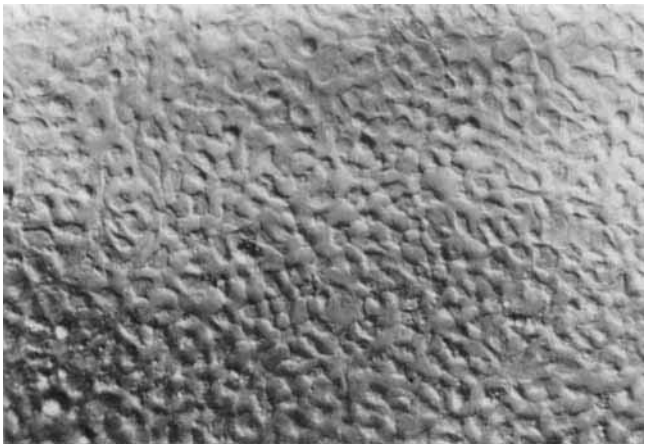

Fig. 13. Meshwork of live neural crest cells observed in situ in a H.H. stage 10 embryo lateral and ventral to the apposed neural folds in the mesencephalic region; note the filopodial contacts between cells. ×640.

1979]. To determine the presence of these epithelial characteristics in the living embryo, we have examined the leading edge neural epithelium in unfixed, live embryos during neural tube closure (Figs. 5, 6). Prior to apposition, large cellular blebs and vesicles were detected in the pulsating neural epithelia (Fig. 6); after closure, small vesicles were observed. Infrequently, a few bridging cells were detected extending from one fold to the other (Fig. 9). Time-lapse cinematography demonstrated that, following the apparently forceful collision between the apposing neural folds, individual cells which had adhered to the opposite cell surface were stretched during occasional occurrences of epithelial retraction. These fold-to-fold cytoplasmic processes probably mark the initial sites of adhesion and were never seen prior to apposition of folds. These observations suggest that "bridging cells" probably do not play a role in the kinetics of neural tube closure. Rather, the large number of filopodia previously observed in the SEM and TEM studies [Bancroft and Bellairs, 1975; Waterman, 1976, 1979] were probably consequent to neuroepithelial shrinkage during fixation and processing, the already attached epithelia being pulled apart. Although we did not detect microvilli, we cannot rule out the possibility that higher magnification studies would have shown them.

Cell Adhesion Molecules

Evaluation of fusing and non-fusing neuroepithelia have shown that an increase in the amount of cell surface coat is associated with neural tube closure [Lee et al., 1978; Smits-Van Prooije et al., 1986]. When the cell surface material had been modified experimentally, the tube failed to close [Lee et al., 1978]. Therefore, it has been suggested that the ability of apposing neural folds to adhere is related to the cell surface coat material [Lee et al., 1978; Takahashi and Howes, 1986; Takahasi, 1988; Thiery et al., 1982]. To begin to identify possible adhesive molecules in the cell surface material during avian neurulation, lectins which bind to specific carbohydrate molecules and antibodies against cell adhesion molecules (N-CAM, L-CAM) have been used [Takahashi and Howes, 1986; Takahasi, 1988; Thiery et al., 1982; Edelman, 1984]. N-CAM and L-CAM were detected throughout the early embryo [Thiery et al., 1982]. Following neural induction, only N-CAM was detected in the neural plate; N-CAM is distributed throughout the neural fold epithelium [Thiery et al., 1982]. The distribution of lectin binding sites and N-CAM antigens on the surface of the neural folds changed as the neuroepithelium progressed from a V-shaped to a C-shaped to a closed tube [Thiery et al., 1982; Edelman, 1984; Takahashi and Howes, 1986; Takahashi, 1988]. On the apical surface of the neural tube, the future region of neural apposition, there was an increase in N-CAM concentration; N-CAM was more diffusely distributed throughout the dorsal region of the closed tube. In the present study, we have investigated the localization of N-CAM during neural tube closure to correlate the distribution of this adhesion molecule to our morphological observations. Our results are similar to those previously seen [Thiery et al., 1982]. A higher concentration of N-CAM was detected on the leading edges of the open neural tube (Fig. 8); following apposition, N-CAM was diffusely distributed throughout the tube (data not shown). The localization of this intense fluorescent stain on the leading edge epithelium supports the hypothesis that the cell surface coat, and N-CAM in particular, probably mediates cell-cell adhesion.

Neural Tube Fusion

Neural tube fusion begins at the site of initial apposition, the mesencephalon (Fig. 10), and progresses both anteriorly and posteriorly. Using the high-definition light microscope, fusion can be identified by the absence of the apposition line (Fig. 10). Several investigators have described the sequence of neural and surface epithelial fusion [Bancroft and Bellairs, 1975; Marin-Padilla, 1970]. Neuroepithelial fusion was shown to lag behind surface epithelial fusion. To determine if technical limitations may have led to this interpretation, we have re-examined the sequence of fusion of the avian neural tube in live embryos. Through-focus into the H.H. stage 10 and 11 embryos permitted concurrent observations of the dorsal (surface) and ventral (neural) epithelia. Our observations have demonstrated that the neuroepithelium (Fig. 11A) fused prior to the surface epithelium (Fig. 11B). The differences between our results and those of others [Bancroft and Bellairs, 1975; Marin-Padilla, 1970] are probably due to our observations having been made on live, developing embryos. It is important to keep in mind that fixatives and processing may often result in distortions of the delicate embryonic tissues and make difficult the distinction between apposition and fusion.

Neural Crest Cell Migration

Neural crest cells migrate from the neural tube throughout the embryo to differentiate into numerous tissues, including the skeleton of the branchial arches, cranial gangli, and the peripheral nervous system [see review Noden, 1988]. Crest cell migration pathways

have been described in the head [Noden, 1975, 1980] and trunk [Erickson, 1988; Loring and Erickson, 1987; Bronner-Fraser, 1987]. In the cranial region, the crest cells migrate lateral to the neural tube, and in the mesencephalic and rhombencephalic regions, in the cell-free space between the neural tube and surface epithelium. This space contains extracellular matrix (e.g., fibronectin, laminin, glycosaminoglycans)[Pratt et al., 1975; see reviews, Erickson, 1987; Bronner-Fraser, 1987], which has been shown to facilitate cell migration [Bronner-Fraser, 1987; Newgreen, 1982; Newgreen et al., 1982; Erickson, 1987, 1988].

To investigate neural crest cell migration, three types of in situ studies have been conducted: 1) disruption of crest cell-matrix interactions [see review Bronner-Fraser and Fraser, 1988], 2) injection of labeled neural crest cells or particles into the embryo [see review Bronner-Fraser, 1987], and 3) mapping of the position of neural crest cells prior to, during, and after migration [Noden, 1975, 1980; Loring and Erickson, 1987; Bronner-Fraser and Fraser, 1988]. In all of these investigations, crest cell migration was observed indirectly in fixed tissues. In order to examine cell migration directly, neural tubes have been explanted and the in vitro migration of crest cells has been determined [see reviews, Erickson, 1987; Bronner-Fraser, 1987]. Based on these in situ and in vitro studies, it has been concluded that avian neural crest cells migrate as sheets of cells [Noden, 1988; Bronner-Fraser, 1987; Erickson, 1988].

Previously, the tracking of migrating crest cells in situ in the living embryo has been nearly impossible. Using a high-definition light microscope and time-lapse cinematography, we have observed in situ a meshwork of synchronously pulsating crest cells which remain in contact with each other via several filopodia (Fig. 13). We have seen a few cells at a time emigrating from the large crest cell population; we have never observed the cells emigrating in large numbers or sheets.

Our results significantly differ from those described previously [Noden, 1988; Erickson, 1988; Bronner-Fraser, 1987]. There are several possible explanations for these differences. First, we must consider the possibility that our observations were made during the wrong developmental time period for major cell migration. To address this concern, we filmed several regions of cephalic crest cells during developmental stages when crest cells have been shown to migrate [Noden, 1975, 1980]. Second, it is also possible that our culture conditions did not permit normal crest migration. This interpretation appears relatively unlikely as we were able to detect extensive neuroepithelial cell movement and neural fold closure under these same conditions. Third, it is possible that the migratory behavior of cephalic crest cells differs from that of trunk cells. Fourth, and the most intriguing, is the possibility that relatively few neural crest cells individually emigrated from the main cell population to colonize distant embryonic areas. Upon reaching their final destination, these "pioneer" cells undergo mitosis and populate these regions with ectomesenchyme. In contrast, most of the crest cells would simply move relatively short distances, if at all, by the passive translocation associated with embryonic growth. This explanation is supported by a recent observation in *Xenopus* embryos [Krotoski et al., 1988]. Using inter- and intra-specific cell markers, Krotoski et al. [1988] investigated neural crest pathways and concluded that neural crest cells migrate as individual cells. Further experimentation is necessary to determine the mode of avian crest cell migration.

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