

# The relationship between emerging neural crest cells and basement membranes in the trunk of the mouse embryo: a TEM and immunocytochemical study

J. STERNBERG\* AND S. J. KIMBER

*MRC Experimental Embryology and Teratology Unit, MRC Laboratories,  
Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK*

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## SUMMARY

The earliest stage of neural crest cell (NCC) migration is characterized by an epithelio-mesenchymal transformation, as the cells leave the neural tube. There is evidence that in a number of cell systems this transformation is accompanied by alteration or depletion of associated basement membranes. This study examines the ultrastructural relationship between mouse NCCs and adjacent basement membranes during the earliest stages of migration from the neural tube. Basement membranes were identified by transmission electron microscopy (TEM) and immunofluorescence using antibodies to type-IV collagen. The ultrastructural features of NCCs and their relationship with surrounding tissues were also examined using TEM. In the dorsal region of the neural tube, from which NCCs originate, the basement membrane was depleted or absent, and with the immunofluorescence technique it was shown that this pattern was reflected in a deficit of type-IV collagen. TEM observations indicated that ultrastructurally NCCs differ from their neuroepithelial neighbours only in overall cell shape and their relationship to other cells and the extracellular matrix.

## INTRODUCTION

Trunk neural crest cells (NCCs) originate along the dorsal midline of the neural epithelium in midgestational mouse embryos (Erickson & Weston, 1983; Sternberg & Kimber, 1986). It is known that chick NCCs subsequently migrate laterally and ventrally into the embryo and differentiate into a variety of tissues including melanocytes and peripheral neurones (for reviews see Le Douarin, 1980; Le Douarin *et al.* 1984). Without specific markers it is not possible to analyse the fates of mouse trunk NCCs once they leave the dorsal surface of the neural tube as they become indistinguishable from surrounding tissues (Sternberg & Kimber, 1986).

Many studies have attempted to define aspects of the extracellular environment that may influence NCC migration and it is known that fibronectin, an important adhesion molecule (Hynes & Yamada, 1982), is present in chick (Mayer, Hay &

\* Present address: Department of Histopathology, St George's Hospital Medical School, Tooting, London, UK.

**Key words:** mouse embryo, neural crest cells, type-IV collagen, basement membranes, immunocytochemistry.

Hynes, 1981; Thiery, Duband & Delouvé, 1982; Newgreen & Thiery, 1980; Duband & Thiery, 1982) and mouse (Derby & Pintar, 1978; Sternberg & Kimber, 1986) NCC pathways. Glycosaminoglycans (chick: Pratt, Larsen & Johnston, 1975; mouse: Derby, 1978), laminin and entactin (Sternberg & Kimber, 1986) are also present. Fibronectin is an effective substrate for both chick (Rovasio *et al.* 1983) and mouse (Sternberg, unpublished observation) NCC migration *in vitro*. Immunolocalization of fibronectin, laminin and entactin has shown that these glycoproteins are present in, or in association with, embryonic basement membranes, which are contacted by NCCs as they migrate (chick: Tosney, 1978, 1982; Bancroft & Bellairs, 1976; Thiery, Duband & Delouvé, 1982; Brauer, Bolender & Markwald, 1985; mouse: Sternberg & Kimber, 1986). Consequently it has been proposed that basement membranes are an important substrate for motile NCCs. During the earlier stages of NCC migration, however, when the cells dissociate from the neuroepithelium in the chick embryo, a change in structure or the total loss of basement membranes appears to be essential for NCC migration to occur (Tosney, 1978, 1982). Similar changes occur in cephalic regions of mouse embryos (Nichols, 1985; Innes, 1985). Loss of a continuous basement membrane has also been considered important in other cell systems where cells acquire a migratory phenotype as they leave an epithelium. This has been demonstrated in the primitive streak region of mouse embryos, using type-IV collagen immunocytochemistry (Herken & Barrach, 1985) and in metastasizing tumours (Ingber & Jamieson, 1982; Liotta, Rao & Barsky, 1984). In the latter system there is evidence for enzymic digestion of basement membrane matrices (Liotta *et al.* 1979; Liotta, Garbisa & Tryggvason, 1982; Biswas, 1982; Woolley, 1982; Pardo, Rosenstein, Montfort & Perez-Tamayo, 1983; Kramer & Vogel, 1984). This study concentrates on the earliest stages of NCC migration when the cells undergo epithelio-mesenchymal transformation, leave the neural tube and migrate over its dorsal surface. The behaviour of emerging mouse NCCs in relation to neural tube-associated basement membrane was examined with the aid of transmission electron microscopy (TEM) and type-IV collagen immunofluorescence. TEM was also used to examine ultrastructural features of NCCs and their environment, which may be related to migration.

## MATERIALS AND METHODS

### *Isolation of embryos*

Random-bred MF1 female mice were naturally mated with B6CB(C57BL/6JLac×CBA/Lac) F<sub>1</sub> hybrid males. Fertilization was assumed to be around the midpoint of the light-dark cycle at 01.00 h. The presence of a vaginal plug the next morning indicated successful mating (the day of vaginal plug was designated day 1 of pregnancy). Embryos were investigated on day 8, 9, 10, 11 and 12 of pregnancy (approximate embryonic age 7½, 8½, 9½, 10½ and 11½ days, respectively). After sacrificing the mice, the decidua were removed and placed either in Hank's balanced salt solution (HBSS) or 0.1 M-phosphate-buffered saline (PBS). PBS was made according to Sørensen's method (Glauert, 1975) and 0.85% NaCl added. The embryos were dissected free of decidua, yolk sac and amnion, and the number of somites noted. For transverse sections embryos were cut into 2–4 pieces perpendicular to the long axis, with watchmaker's forceps (see

Sternberg & Kimber, 1986). The somite level of each dissected portion was noted. Embryos were left intact for longitudinal/sagittal sections.

### *Preparation of embryos for immunofluorescence*

The preparation of embryos for immunofluorescence has been described in detail previously (Sternberg & Kimber, 1986). Briefly, embryos were fixed in 4% paraformaldehyde in PBS. The tissue was washed and incubated sequentially at 37°C in PBS containing 5% sucrose, 15% sucrose and 15% sucrose plus 7% gelatin. The embryos were transferred to plastic moulds in fresh gelatin and allowed to set. For transverse sections, embryos were oriented so that the approximate axial level of sections could be established. Gelatin blocks were then mounted on cork with OCT (Tissue Tek) and snap frozen in hexane cooled in liquid nitrogen. Longitudinal and transverse sections were cut at 7–10 µm in a cryostat at –20 to –30°C. Sections were mounted on slides coated with 1% gelatin and stored frozen until required for staining.

### *Type-IV collagen immunofluorescence*

The antibody to type-IV collagen (rabbit anti-mouse type-IV collagen) was a gift kindly donated by Dr M. Warburton (Ludwig Institute, Sutton, UK). The antibody was prepared from EHS sarcoma basement membrane and affinity purified. It did not cross react with type-I, -II and -III collagen or basement membrane glycoprotein (Liotta *et al.* 1979a). Serial dilutions of the antibody were tested to determine the most suitable concentration. It was finally used at a dilution of 1:70 or 1:50 in 0.5% or 1% Tween (TWEEN 20, Sigma) in PBS. Normal rabbit serum was used at the same concentrations for the nonimmunoreactive control. The second antibody, goat anti-rabbit IgG, conjugated to fluorescein isothiocyanate (FITC) (Miles-Yeda, Israel), was used at a concentration of 1:50 in 0.5% Tween in PBS.

Throughout the staining procedure, the microscope slides were maintained in humidified Petri dishes. Sections were initially washed with 0.5% Tween in PBS for 10–20 min, followed by a 10 min incubation in 2% glycine in PBS. After a further wash in PBS, sections were incubated in 10% heat-inactivated normal goat serum in PBS (HINGES) for 10 min. Aliquots of 50 µl of type-IV collagen antibody or normal rabbit serum were pipetted onto the sections which were incubated for a period of 1–1½ h. Sections were then washed again for approximately 10 min and incubated in HINGES for another 10 min, before addition of 50 µl aliquots of the FITC-conjugated 2nd antibody. Sections were incubated for 30 min–1 h, washed finally for up to 30 min in PBS and mounted in 'Uvinert' mountant (Gurr) under No. 1 coverslips. Sections were viewed with a Leitz Ortholux microscope, using excitation filter BP450-490 (1/2) and suppression filter LP515, and photographed on Ilford HP5 film.

### *Preparation of embryos for TEM*

Embryos of the same age as those used for immunofluorescence (except for 7½ day embryos) were dissected free from decidua and extraembryonic membranes in HBSS and transferred to primary fixative for 1 h at room temp. Primary fixative consisted of 2.5% glutaraldehyde, 1% paraformaldehyde, 0.05% KFeCN, 2 mM-CaCl<sub>2</sub> in 0.075 M-sodium cacodylate buffer. In some cases the paraformaldehyde was left out of the fixative, and either 0.5% cetyl pyridinium chloride (CPC) or 2% tannic acid was added. For embryos processed with ruthenium red (Luft, 1971), the primary fixative consisted of 0.075 M-sodium cacodylate, 0.05% ruthenium red and 1.2% glutaraldehyde. Embryos were incubated for 1 h, washed in buffer and transferred to the secondary fixative, consisting of 0.66% OsO<sub>4</sub>, 0.075 M-sodium cacodylate and 0.05% ruthenium red, for overnight incubation. After fixation, the material was washed overnight in 0.075 M-cacodylate buffer containing 2 mM-CaCl<sub>2</sub>. Final fixation for 1 h in 1% OsO<sub>4</sub> in cacodylate buffer was followed by extensive washing in distilled water. The embryos were then block stained for 2 h in 1% uranyl acetate in distilled water and washed again in distilled water. After dehydration, the embryos were transferred from 100% ethanol *via* propylene oxide to Epon resin (Coulter, 1967). Semithin (1 µm) sections were cut with glass knives on a Sorvall ultramicrotome and stained with 1% toluidine blue. Thin sections (50–100 nm), cut with a GEFERI diamond knife, were mounted on 300-mesh grids and stained with 1% uranyl acetate and 1% lead citrate. Sections were viewed on a Jeol electron microscope and photographed on Ilford EM film.

## RESULTS

*Distribution of type-IV collagen**7½ day embryos*

The most intense staining with the antibody to type-IV collagen is seen in Reichert's membrane, the basement membrane of the parietal endoderm. It also stains the basement membrane separating the visceral endoderm from both the embryonic and extraembryonic ectoderm. In longitudinal sections, staining of the distal embryonic regions is reduced and is markedly absent in the region where the primitive streak is forming. When mesoderm has penetrated between the original germ layers, type-IV collagen is present between the mesoderm and both endoderm and ectoderm, as demonstrated by others (Leivo, Vaheri, Timpl & Wartiovaara, 1980; Herken & Barrach, 1985) (not shown here).

*8½ day embryos*

In early 8½ day, neural groove-stage embryos, the only type-IV collagen staining apart from that of the extraembryonic membranes is in the basement membrane between the neural plate and underlying lateral mesoderm (Fig. 1A). At this stage and later, when the neural folds are significantly elevated, staining extends throughout the length of the neural fold basement membrane, but the intensity appears reduced at the crests of the folds (Fig. 1B). After fusion, the dorsal region of the neural fold shows reduced staining.

*9½ day embryos*

The epidermal ectoderm covers the dorsal aspect of the embryo in apposition to the somites and dorsal neural tube. It is a flat, squamous epithelium, sitting on a basement membrane. Strong type-IV collagen staining is present throughout this basement membrane in many embryos, and in all embryos staining is present in those regions overlying the somites (Fig. 1C). In some embryos at the same stage, staining is markedly weak or absent in the basement membrane where the epidermis covers the neural tube. The dorsal aortae and the gut epithelium are delineated by basement membranes stained for type-IV collagen. The notochord is also surrounded by a layer containing type-IV collagen.

Cells within the pseudostratified epithelium of the neural tube are devoid of type-IV collagen which is also absent among lateral mesoderm cells. In the pre-somitic mesoderm and later in the forming somites some type-IV collagen is associated with the cells. Where dermamyotome and sclerotome are forming, some fibrillar stain is seen between sclerotomal cells. Longitudinal sections revealed the presence of type-IV collagen in basement membranes of discrete somites.

At all axial levels of 9½ day embryos, type-IV collagen staining is intense in the ventrolateral basement membrane of the neural tube. In the head, and anterior to somite 5 (approximately) this staining extends into the dorsal basement membrane

directly under the presumptive epidermis (Fig. 1E). At more posterior levels of the embryo, however, staining in the dorsal basement membrane of the neural tube is very weak or absent (Fig. 1C,D). In some cases this is mirrored by weak staining of the adjacent epidermal basement membrane. This can be seen clearly in longitudinal sections (Fig. 1F). This phenomenon extends along the major part of the postvagal neural tube, whether viewed in transverse or longitudinal sections. In whole embryos, which are 'U'-shaped, sections that include both anterior and posterior regions of embryo showed staining in anterior dorsal neural tube basement membrane and lack of staining in posterior regions. This confirms that the results described above are not an artefact caused by differences in staining between sections. Apart from the absence of staining in the basement membranes of the dorsal neural tube the overall staining pattern was similar to that seen previously with antibodies to fibronectin, laminin and entactin (Sternberg & Kimber, 1986). In other embryonic regions examined, no significant variation of staining with type-IV collagen was noted.

#### *10½ day embryos*

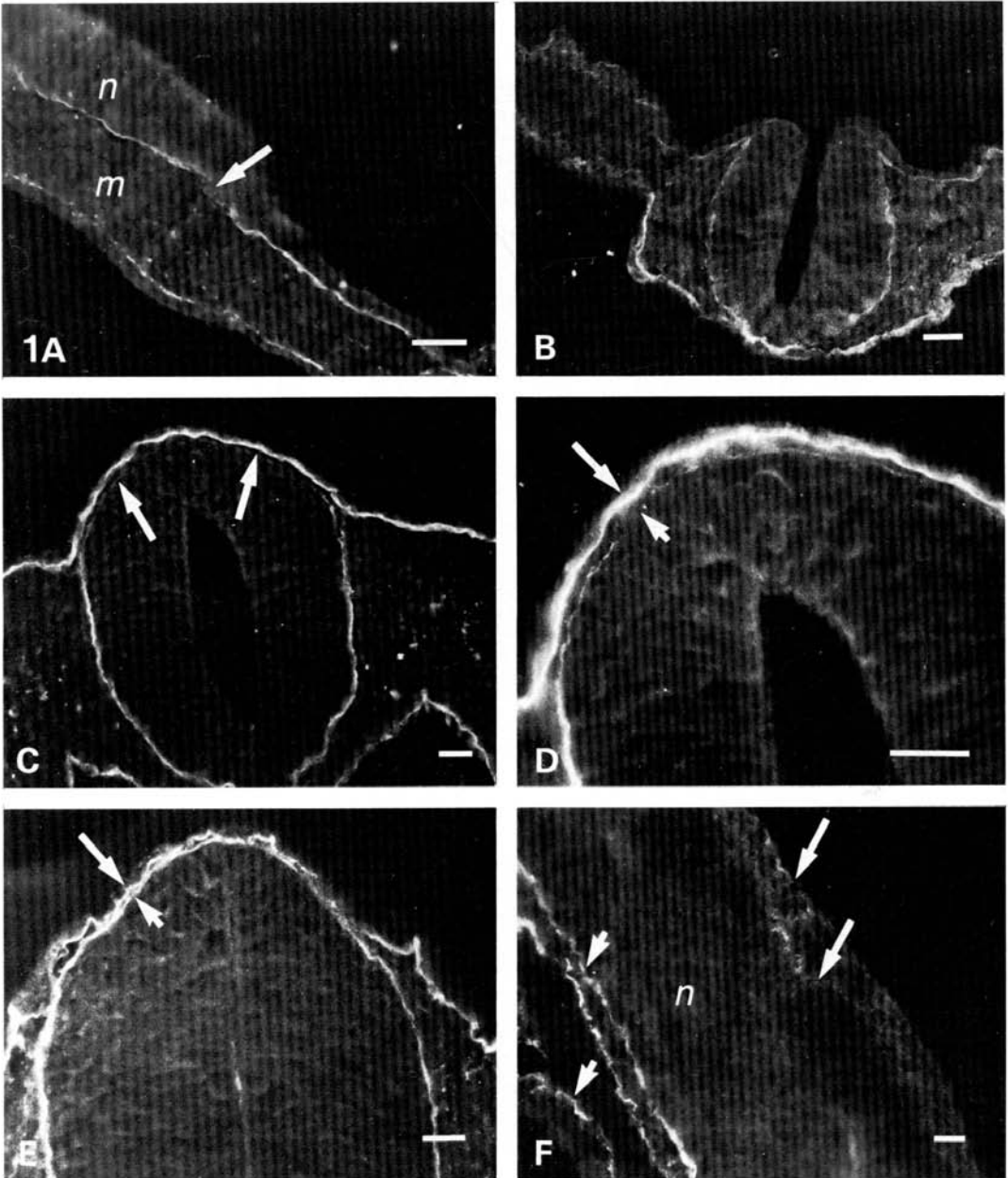
Type-IV collagen staining is present in basement membranes at a similar intensity to that seen in 9½ day embryos, and the overall pattern is very similar (Fig. 2A). Basement membranes of structures such as the neural tube and aorta are strongly stained, some stain was seen among somitic cells, but none within epithelia. At this stage of embryonic development, capillaries are forming and are well delineated using this antibody. The dorsal basement membrane of the neural tube still lacks type-IV collagen staining in regions posterior to somite 10 (approx.) but more anteriorly limited regions of fluorescent staining are seen in areas where they were absent in 9½ day embryos. This staining is punctate consisting of short, narrow, discontinuous flecks parallel to basal surfaces of neuroepithelial cells. At yet more anterior regions, and in the head, staining of dorsal neural tube basement membrane is continuous.

#### *11½ day embryos*

In embryos of this stage, the majority of type-IV collagen is located in the same structures as in the younger embryos, at approximately the same intensity. By this stage, at most axial levels of the neural tube, the dorsal region of the neuroepithelium and the overlying ectoderm are separated by one or more layers of cells (Fig. 2B). These cells are mesenchymal, and are sometimes interspersed by blood vessels containing red blood cells. There are also type-IV collagen-lined capillaries in the neural tube. At all axial levels of the neural tube examined, the basement membrane in the dorsal region shows strong staining for type-IV collagen, unlike that seen in earlier embryos (see Fig. 1C,D). In some cases this staining is closely associated with small blood vessels such that the two cannot be distinguished at the magnifications possible with this technique.

*Morphology and ultrastructure of NCC and neuroepithelial cells**Premigratory regions of neural tube*

Embryos  $9\frac{1}{2}$  days old, with 20–30 somites, were examined by TEM. In caudal regions of the neural tube, where it is flanked by presomitic mesoderm, the neuroepithelial cells are a homogeneous population forming a pseudostratified epithelium. When the neural tube was fractured transversely and viewed by SEM the cells were found to be interdigitated (Sternberg & Kimber, 1986). This was



confirmed with TEM (Fig. 3A). Cell division occurs at the apical (juxtaluminal) surface, from the location of mitotic figures seen by LM and TEM. Nondividing cells were narrow at the apical end, with the nucleus located centrally or basally. At this level of the neural tube the basal surfaces of the dorsal cells (some of which are presumptive NCCs) are closely apposed to the overlying epidermal ectoderm cells. Some of these surfaces are flat, others are curved in a lateral orientation. Few cellular processes extend from the cells. Occasionally, the cells interdigitate closely at their dorsal extremes (Fig. 3B).

Neuroepithelial cells (including presumptive NCCs) are very tightly packed with adjacent cell membranes in close contact (Fig. 3C) and many focal contacts between cells. At their apical ends cells are in contact by junctional complexes including gap junctions (Fig. 3D), adhering junctions and probably tight junctions (Fig. 3E).

The cytoplasm of the neuroepithelial cells contains large numbers of free ribosomes and polyribosomes (Fig. 3B) and some are seen in association with endoplasmic reticulum. Endoplasmic reticulum is more frequently present in its smooth form, distributed at random in the cytoplasm, and very seldom seen in large aggregates of parallel cisternae. Golgi complexes and associated vesicles were occasionally seen (not shown). Mitochondria are distributed throughout the cytoplasm. Cell nuclei occupy most of the width of the cells, and the cytoplasm and its organelles are mainly restricted to apical and basal extremes.

Basement membrane surrounds the ventrolateral neural tube, but very little or no basement membrane is associated with dorsal neuroepithelial cells (Figs 3A, 4A). Conversely, epidermal ectoderm is associated with a strongly delineated basement membrane (Fig. 4A), although in some specimens it is discontinuous.

Fig. 1. The distribution of type-IV collagen shown by immunofluorescence in 8–9½ day embryos.

(A) Neural plate of an 8–8½ day embryo. Type-IV collagen staining is more diffuse in the region of the neural ectoderm/epidermal ectoderm junction (arrow). *n*, neuroepithelium; *m*, mesoderm; ×350; bar, 20 μm.

(B) Transverse section through forming neural tube of an 8–8½ day embryo. Epidermal ectoderm and neural tube basement membranes are stained. This stain is very weak at the tips of the neural folds. ×260; bar, 20 μm.

(C) Transverse section of a 9½ day embryo at the level of the presomitic mesoderm (prior to NCC migration). The epidermal basement membrane is strongly stained, as well as the basement membranes of the aortae, notochord and ventrolateral neural tube. Staining of the basement membrane of the dorsal neural tube is negligible (arrows). ×215; bar, 20 μm.

(D) Higher magnification of C, illustrating the intense stain in the epidermal basement membrane (large arrow) and the weak stain of the dorsal neural tube (small arrow). ×540; bar, 20 μm.

(E) Transverse section through somites 3–4 of an embryo of the same stage as C and D. Staining of the dorsal neural tube basement membrane (small arrow) is of similar intensity to that of the epidermal basement membrane (large arrow). ×286; bar, 20 μm.

(F) A longitudinal section of a small area of a 9½ day embryo. The notochord and ventral neural tube basement membranes show strong staining (small arrows). The dorsal neural tube and epidermal ectoderm show very weak staining (large arrows). *n*, neural tube; ×211; bar, 20 μm.

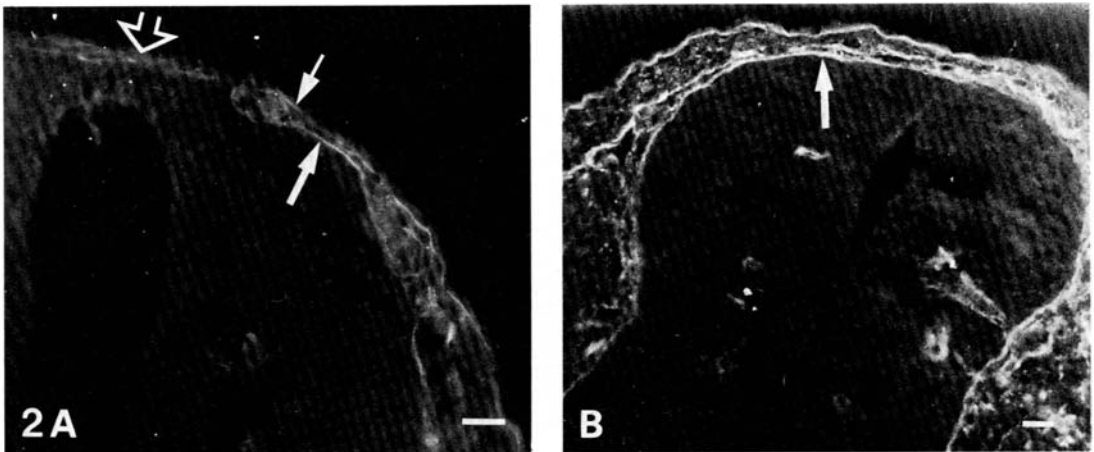


Fig. 2. The distribution of type-IV collagen shown by immunofluorescence in  $10\frac{1}{2}$  and  $11\frac{1}{2}$  day embryos.

(A) Transverse section through the neural tube of a  $10\frac{1}{2}$  day embryo. Type-IV collagen is present in basement membranes of epidermal ectoderm (small arrow) and neural tube (large arrow). Staining is still weak at the dorsal midline (open arrow).  $\times 250$ ; bar,  $20\ \mu\text{m}$ .

(B) Transverse section through the neural tube of an  $11\frac{1}{2}$  day embryo. By this stage strong staining is present around the entire neural tube (note the staining in the dorsal region (arrow)).  $\times 188$ ; bar,  $20\ \mu\text{m}$ .

Addition of ruthenium red during fixation resulted in the retention of more basement membrane and associated material, but did not alter its relative distribution with respect to dorsal neuroepithelium and overlying ectoderm. CPC and tannic acid did not affect the appearance of extracellular matrix material.

#### *Midmigratory regions of neural tube*

The neuroepithelial phenotype retains a fairly constant morphology along the anteroposterior axis of the neural tube, although the epithelium becomes multi-layered and columnar in more anterior regions of the embryo. However, in the extreme dorsal regions cells undergo characteristic changes in morphology and position that form the basis for their identification as NCCs. The changes are gradual, with no abrupt demarcations. The dorsal cells undergoing the transition to the migratory phenotype tend to have their basal ends oriented laterally, away from the midline of the neural tube. As emigration proceeds the leading basal edge protrudes between ectoderm and underlying neuroepithelial cells, as a broad cell process (Figs 4C, 5B). These processes sometimes contain dense parallel arrays of microfilaments (Fig. 5B,C). Smaller cell processes are also seen dorsally and laterally, and these often make contact with the epidermis or its basement membrane or with neighbouring NCCs or neuroepithelial cells (Fig. 5B). As the leading edges of the cells extend laterally towards the somites, the cells become progressively detached from their neighbours and gaps are apparent between them



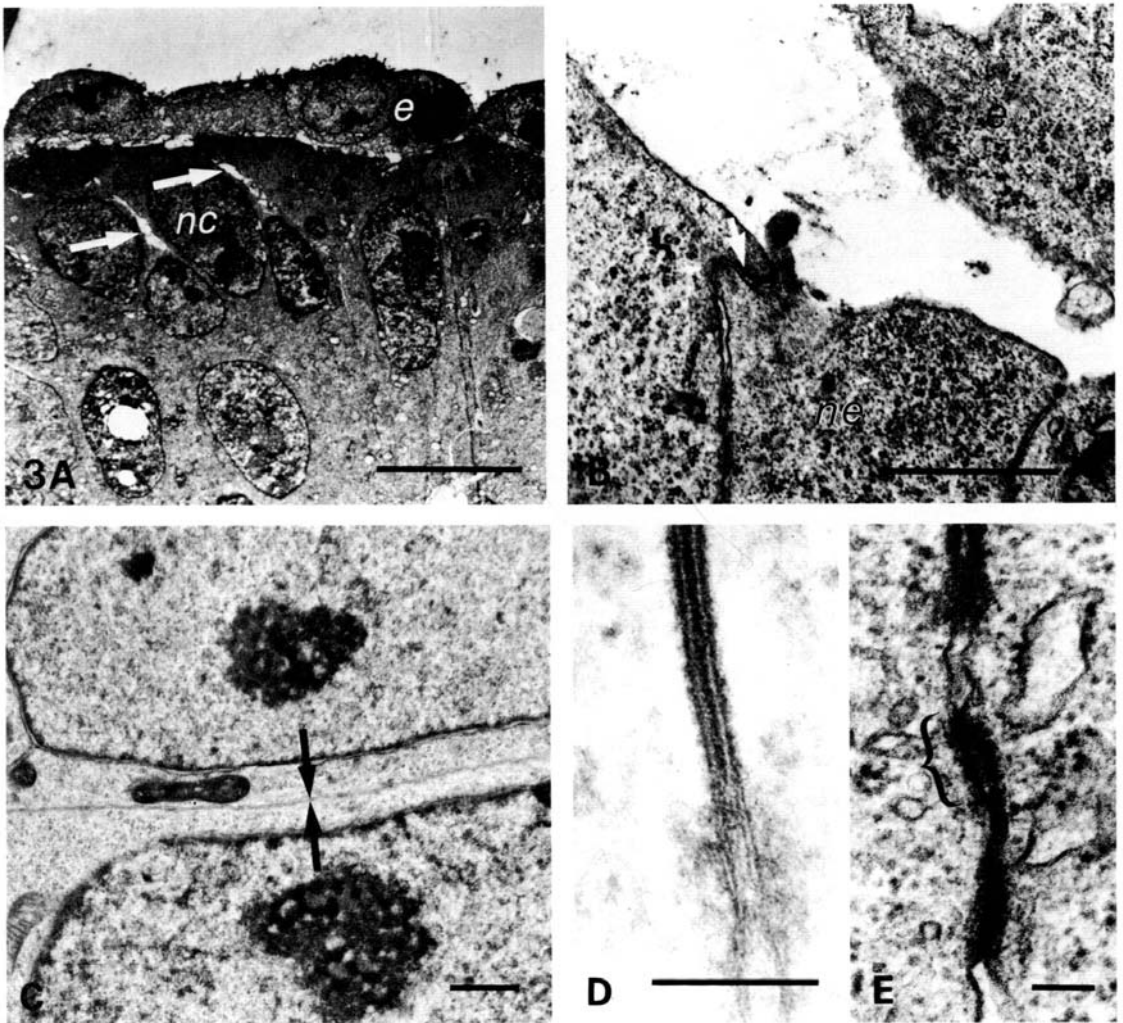


Fig. 3. TEM micrographs of the neuroepithelium of 9½ day embryos.

(A) A transverse section through the posterior region of the embryo prior to overt NCC emigration. The cells are elongated and tightly packed, especially in the central and apical regions, but in the basal region presumptive NCCs are beginning to lose intercellular adhesions (arrows) and assume a lateral orientation. *e*, presumptive epidermis; *nc*, presumptive NCC; ×18 000; bar, 1 μm.

(B) Prior to individualization of NCCs dorsal neuroepithelial cells have blunt basal surfaces, where close interdigitations are sometimes seen (arrow). *e*, presumptive epidermal cell; *ne*, neuroepithelial cell; ×21 000; bar, 1 μm.

(C) Dorsal neuroepithelial cells, prior to and subsequent to NCC emigration, and ventrolateral neuroepithelial cells are in close contact with their neighbours along most of the cell length. This micrograph illustrates the close apposition of two such cells. Specialized junctions are rarely seen. Arrows indicate the juxtapposition of the two cell membranes. ×91 000; bar, 0.1 μm.

(D) A gap junction which is seen typically at or near the apical surface of the neuroepithelium. ×210 000; bar, 0.1 μm.

(E) A junctional complex of the type seen between neuroepithelial cells bordering the lumen. Adhering junctions are most commonly seen (bracket). ×79 000; bar, 0.1 μm.

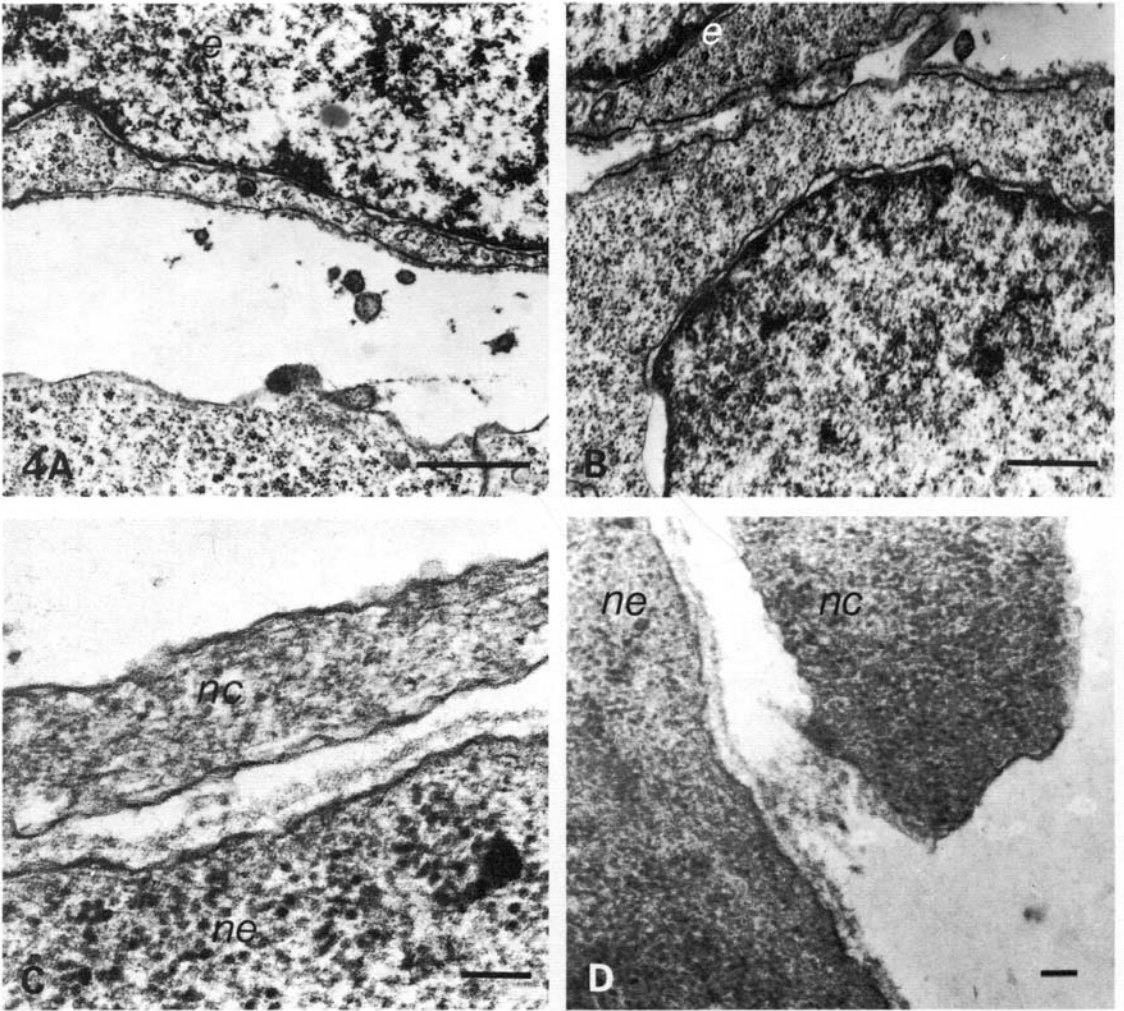


Fig. 4. Basement membranes associated with neuroepithelial cells and NCCs.

(A) A dorsal neuroepithelial cell in the posterior region of a  $9\frac{1}{2}$  day embryo, and an overlying presumptive epidermal cell. Note the basement membrane associated with the epidermal cell. The neuroepithelium is devoid of basement membrane in this region. *e*, epidermis;  $\times 24\,000$ ; bar,  $0.5\ \mu\text{m}$ .

(B) In anterior regions of  $9\frac{1}{2}$  day embryos, both epidermis and neuroepithelium have basement membranes. *e*, epidermis;  $\times 14\,000$ ; bar,  $1\ \mu\text{m}$ .

(C) A lamellipodium of an NCC which has left the neural tube and is migrating along its dorsolateral surface. The lamellipodium is close to and parallel to the basement membrane. *nc*, neural crest cell; *ne*, neuroepithelial cell;  $\times 86\,000$ ; bar,  $0.1\ \mu\text{m}$ .

(D) The tip of the lamellipodium of an NCC migrating along the lateral region of the neural tube is contacting the basement membrane of the basal surface of a neuroepithelial cell. Extracellular material is interposed between the cell process and the basement membrane. *nc*, neural crest cell; *ne*, neuroepithelial cell;  $\times 42\,000$ ; bar,  $0.1\ \mu\text{m}$ .

(Fig. 5A,B). The apical ends of the cells tend to remain within the neuroepithelium until further migration has occurred. Early migrating NCCs that have emerged from the neural tube are often flattened elongated cells, with a large central nucleus, although some are more stellate. They are indistinguishable from neuroepithelial cells in the distribution and density of organelles such as mitochondria, ribosomes and endoplasmic reticulum. When more than one NCC is

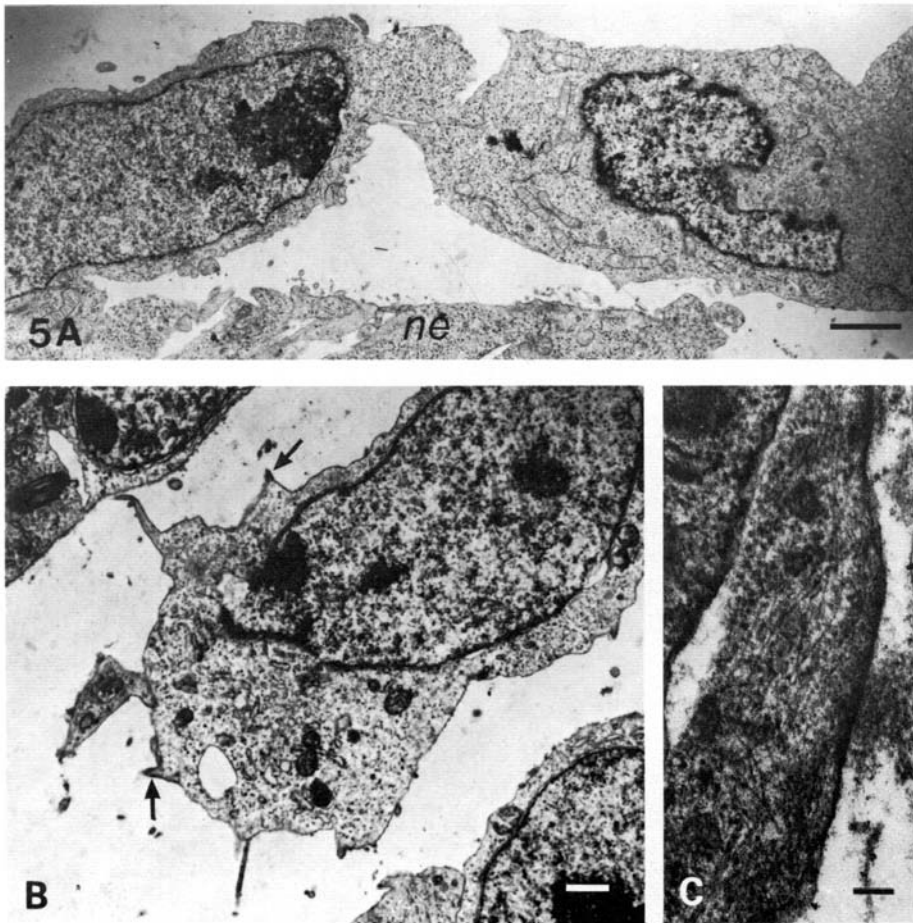


Fig. 5. TEM micrographs of migrating neural crest cells.

(A) Two NCCs on the dorsal surface of the neural tube. The dorsal midline is to the left, the cells are presumed to be moving to the right. The more advanced cell is making no contact with the neuroepithelium. The second cell still retains intimate contact with the neuroepithelium at its trailing edge (far left of micrograph). Note the close association between the two NCCs. *ne*, neuroepithelium;  $\times 4300$ ; bar,  $0.2 \mu\text{m}$ .

(B) A NCC is migrating between neuroepithelium (bottom right) and epidermis (top left). It is making close contact with the epidermal basement membrane with a fine cell process. Parts of other cell processes are also seen (arrows).  $\times 5400$ ; bar,  $1 \mu\text{m}$ .

(C) A high magnification micrograph illustrating microfilaments seen in a NCC process. This cell process is in close association with amorphous extracellular material, which it appears to contact *via* an electron-dense membrane plaque.  $\times 40\,000$ ; bar,  $0.1 \mu\text{m}$ .

migrating across the neural tube surface they are frequently in very close contact with each other, with the trailing edge of one cell being overlapped by the leading edge of the following cell (Fig. 5A). However, specialized junctions between these cells were not observed.

At this stage of migration little basement membrane is associated with neural crest cells or dorsal neuroepithelial cells, although the overlying ectoderm and ventrolateral neural tube have marked basement membranes (Fig. 4D). The addition of ruthenium red during fixation did not change the relative distribution of basement membrane material.

#### *Postmigratory regions of neural tube*

At anterior levels of trunk neural tube and in head regions (of 9½ day embryos) migration of NCCs from the neuroepithelium has ceased. Cell processes no longer extend from the dorsal neural tube and the population of neuroepithelial cells is increasingly homogeneous. The basal surfaces of dorsal cells are no longer tapering, but are now flat and together form a continuous dome over the dorsal neural tube. Two ultrastructural changes were observed in this region; first, large numbers of mitochondria appeared, particularly in the basal cytoplasm (not shown). Second, basement membrane material appears as a fairly thick continuous layer over dorsal regions of the neuroepithelium (Fig. 4B) so that the neural tube is now completely surrounded by a basement membrane.

Flattened mesenchymal cells were seen over dorsolateral regions and also along the sides of the neural tube. By extrapolation from our SEM observations these are probably NCCs in various stages of migration. In these populations of NCCs cell surfaces tended to be flattened against the basement membrane of the neural tube. Lamellipodia and occasional filopodia also make close contacts with basement membranes, and additional extracellular material was sometimes located between regions of the lamellipodia and the basement membrane (Fig. 4D). NCCs sometimes exhibited electron-dense plaques next to their plasma membrane in regions of contact with basement membrane (not shown).

#### DISCUSSION

This study describes the ultrastructure of mouse NCCs emerging from the dorsal neuroepithelium in the trunk and in particular their relationship to components of the basement membrane of the neural tube. Immunocytochemistry using antibodies to type-IV collagen showed that this protein is greatly reduced or even absent in the dorsal region of the neural tube prior to, and at the stage of, NCC migration in 9½ day mouse embryos. Fluorescent staining for type-IV collagen was present at anterior axial levels and in older, 10½ day and 11½ day embryos, in which most neural crest cells have left the neural tube. There was good correlation between areas of the dorsal neural tube that lacked type-IV collagen staining and the absence of a definitive basement membrane at the ultrastructural level. Neural crest cells could be clearly distinguished by TEM as they emerged from the neural

tube. Their ultrastructure is similar to that of neuroepithelial cells except that they contain parallel arrays of microfilaments in lamellipodia.

There are other instances in embryonic development where, as during the emergence of NCCs, a lack of type-IV collagen is found adjacent to cells undergoing morphogenetic movements. In particular, type-IV collagen and basement membrane material are absent or reduced in the region of primitive streak where primary mesoderm is emerging (Herken & Barrach, 1985). Remodelling of basement membrane with disappearance of type-IV collagen staining also occurs during the branching of salivary glands and other organs (Bernfield, Banerjee, Koda & Rapraeger, 1984), and the development of the lens capsule (Linsenmayer, Fitch & Mayne, 1984). Furthermore, during formation of the optic sulcus in the rat embryo it has also recently been reported that the basement membrane is incomplete (Tuckett & Morriss-Kay, 1986).

A complete basement membrane in the dorsal region of the neural tube would present a barrier to emergence of the NCCs, thus it is tempting to connect the lack of this structure with the localized emergence of NCCs. However, type-IV collagen, an integral basement membrane component (Kefalides, 1975; Linsenmayer *et al.* 1984; Timpl, 1985), is lacking from the dorsal neural tube well before emergence of NCCs. This suggests that the absence of this structure does not directly trigger the migration of NCCs or release them from a previous restraint.

The basement membrane of the neuroepithelium has also been reported to be missing in the head region during mouse NCC migration (Innes, 1985) and it is similarly absent or severely reduced in the chick embryo (Newgreen & Gibbins, 1982), although in other reports in rodents (Nichols, 1985; Erickson & Weston, 1983) and the chick (Tosney, 1978, 1982) only local disruption of the basement membrane was described. All these studies do indicate that an alteration and/or reduction of basement membrane material is a consistent and possibly essential accompaniment to NCC emergence. The apparent lack of basement membrane material may occur for a variety of reasons. Type-IV collagen immunofluorescence might be absent because the antigen is masked by other components (Linsenmayer *et al.* 1984), but this seems unlikely since ultrastructurally a basement membrane is not visible. Type-IV collagen might be degraded in the dorsal region of the neural tube as during the metastasis of certain tumours (Liotta *et al.* 1979a,b, 1980, 1984) by the action of specific collagenases (Biswas, 1982) acting *in situ* (Woolley, 1982). While in other tumours there is no correlation between metastatic capacity and collagenase type-IV activity, cell content and secretion of plasminogen activator have been positively correlated with metastatic capacity (Nicolson, 1984; Eisenbach, Segal & Feldman, 1985a,b). A role for proteolytic degradation in emergence of chick neural crest cells involving plasminogen activator has been suggested previously (Le Douarin, 1984; Valinsky & Le Douarin, 1985). However, in this study we observed that younger mouse embryos, at the time of neural tube fusion and subsequent stages, lacked type-IV collagen in the dorsal neuroepithelium, suggesting that some components of the basement membrane are not synthesized or secreted in the dorsal region of the neural tube until

after the emergence of NCCs. It would be expected that the absence of a dorsal neural tube basement membrane, described here, would be mirrored by loss of laminin and entactin from this region since these extracellular matrix components have been found almost exclusively in basement membranes (Timpl, 1985), particularly in the lamina densa (Laurie & LeBlond, 1983; Meier & Drake, 1984). In a previous paper, we described the relatively continuous distribution of these components over the dorsal neural tube. It therefore appears that emergence of individual NCCs does not disrupt the fluorescent staining for laminin and entactin in thick sections to the same extent as type-IV collagen staining. However, small disruptions in staining were seen in some sections, particularly those in which GAGs were demonstrated with Alcian blue (fig. 3A,B, Sternberg & Kimber, 1986). The staining for laminin and entactin is probably due to the electron-dense material and fragments of basement membrane that are seen in contact with the dorsal neuroepithelium and NCCs during their emergence. It is also possible that noncollagenous components are loosely organized in the absence of type-IV collagen and are not resolved in the electron microscope. A minimum concentration of type-IV collagen together with laminin, for example, may be necessary for the formation of a supramolecular basement membrane complex (Martin *et al.* 1983). It was also noted in the results that some 9½ day embryos appear to have reduced type-IV collagen staining and a discontinuous basement membrane beneath the epidermis in posterior regions of the embryos. This may be explained either by the action of collagenolytic enzymes (Biswas, 1982; Woolley, 1982) or by the fact that the ectoderm has only recently become continuous over the newly formed neural tube and a complete basement membrane, including type-IV collagen, had not yet been assembled in some embryos (as discussed above).

The ultrastructural changes in the NCCs as they convert from an epithelial to a mesenchymal phenotype are of considerable importance for the process of emergence from the neural tube. These include alteration in cell shape, the development of cell processes, organized cytoskeletal elements and focal contacts, and the disappearance of intimate contact along the lateral faces of adjacent cells. These are characteristics associated with conversion from nonmotile to motile behaviour (Heasman & Wylie, 1983). Similar changes occur in the formation of NCCs in amphibia (Löfberg, Ahlfors & Fallstrom, 1980) and birds (Tosney, 1978, 1982; Bancroft & Bellairs, 1976) and have been reported for mouse NCCs by others (Innes, 1985; Erickson & Weston, 1983). Indeed there are striking similarities with other cells undergoing parallel phenotypic transitions, such as metastatic tumour cells (Liotta *et al.* 1982) and primitive streak mesoderm (Solursh & Revel, 1978; Spiegelman & Bennett, 1974; Spiegelman, 1976). As they emerge from the neural tube the contact between adjacent cells diminishes and the cells lose close cell-cell adhesion. In avian embryos loss of cell adhesion molecules such as N-CAM (Thiery *et al.* 1982) or a different, calcium-dependent adhesion molecule (Newgreen & Gooday, 1985) may be required for the initial release of NCCs.

Finally, cells were seen to make frequent contact with basement membrane and other extracellular matrix material as they emerge. Contact was observed along both cell processes and cell bodies and sometimes occurred *via* electron-dense plaques (see also Ebendal, 1977). Fibronectin is known to be a constituent of the extracellular matrix in the early crest pathway of the mouse (Sternberg & Kimber, 1986), and in the chick it is known to interact with cell surface receptors on NCCs, promoting migration *in vivo* (Bronner-Fraser, 1985; Duband *et al.* 1986). It seems likely that mouse NCCs are also in intimate contact with fibronectin in their vicinity which may modulate their shape and behaviour through interaction with the cytoskeleton (Lazarides & Burridge, 1975; Hynes & Destree, 1978; Horwitz *et al.* 1986). The presence of a continuous distribution of fibronectin around the neural tube prior to and during NCC migration (Sternberg & Kimber, 1986) is consistent with its importance from the initial stages of emergence.

In conclusion, this study clearly demonstrates that type-IV collagen and a definitive basement membrane are lacking over the dorsal neural tube until after the emergence of neural crest cells in the trunk of the mouse embryo. Ultrastructural observations suggest that a number of factors such as disappearance of cell-cell adhesions and junctional complexes, the mobilization of the cytoskeleton and the adoption of a 'migratory' phenotype (Heasman & Wylie, 1983) contribute to the transformation from epithelium to neural crest mesenchyme. The absence of an organized basement-membrane barrier rich in type-IV collagen must facilitate the emergence of the newly formed NCCs from the neural tube and may be a crucial factor in their localized emergence solely from the dorsal segment of the tube.

The authors thank Ms K. Hughes for typing the manuscript.

J. Sternberg was funded by an MRC research studentship and this work was carried out in partial fulfilment of the PhD degree.

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(Accepted 4 August 1986)