Neural crest formation in the head of the mouse embryo as observed using a new histological technique

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SUMMARY

A histological technique is described which results in the differential staining of neural crest cells. This is used to describe the formation and early migration of crest cells in the head of the mouse embryo. The first indications of crest formation are seen in the midbrain/anterior hindbrain at 3-4 somites where crest cells accumulate in the basal surface of the ectodermal epithelium near the future margin of the neural plate. Shortly thereafter (4-6 somites) these cells disrupt the basal surface of the epithelium and escape as mesenchyme. The apical epithelial cells in this region become the surface ectoderm adjacent to the neural plate. Subsequently, crest is formed from neural plate rather than surface ectoderm. In addition, mesenchyme is formed from presumptive surface ectoderm in a groove in the lateral portion of the fold between the forebrain and the midbrain. By 5-7 somites, crest mesenchyme is formed at all levels of the midbrain, hindbrain, and from the margins of the forebrain adjacent to the optic pits. Because of the bending of the embryonic axis, forebrain crest cells appear to migrate dorsally over the presumptive eye where they are met by ventrally migrating midbrain crest cells. Crest formation continues in the region of the midbrain and hindbrain during, and for an undetermined period after closure of the head folds at between 8 and 16 somites.

These results demonstrate differences in the origin and timing of crest formation between chick and mouse. From this may be inferred different patterns of crest migration as well.

In addition, the ability to directly observe early crest formation should aid in the analysis of the mechanisms by which epithelial cells are converted into mesenchyme.

INTRODUCTION

Neural crest cells arise from the transient conversion of epithelial cells near the margins of the neural plate into mesenchyme. Little more is known of this process because it is difficult to distinguish forming crest cells from the other cell types which surround them. Interpretation of conventional histological sections has resulted in controversy regarding several aspects of this process. Crest cells may or may not form from presumptive surface ectoderm (Horstadius, 1950; Hill & Watson, 1958; Verwoerd & van Oostrom, 1979). Similarly there
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is disagreement on whether or not crest cells form in the forebrain region of various embryos. In the chick, crest cells form from the caudal-most forebrain, but not that region of the forebrain from which the eye will form (Johnston, 1966; Noden, 1978a, b; Di Virgilio, Lavenda & Worden, 1967). In mammals, Celestano da Costa (see Adelman, 1925), looking at the guinea pig and Bartelmez (1962), looking at the rat, identified forebrain crest formation associated with the presumptive eye while Adelman (1925), looking at the rat and Halley (1955) looking at the cat, did not.

In spite of these controversies some agreement does exist on the crest formation in the head of the mammalian embryo. First of all, formation begins at the margins of the midbrain and subsequently spreads to the hindbrain (Adelman, 1925). With respect to the topography of the embryo, crest formation and migration in the head of the mouse is underway well before the neural folds approach and fuse (Johnston & Listgarten, 1972). Similarly, flexures of the neural plate begin prior to tube formation. This may be contrasted with the chick where neither crest migration nor plate flexure begins until the neural folds have begun to close. Scanning electron microscopy of the surface of the head folds in the mouse and hamster (Waterman, 1976) reveals a homogeneous epithelium which subsequently segregates into regions composed of two distinct cell types: the larger flattened cells of the surface ectoderm and the cobblestone epithelium of the neural plate. The boundary zone between these two regions was shown to be associated with neural crest formation.

Several authors have used histological techniques which aid in distinguishing crest cells from other cell types. Stone (1932) noted that crest cells in *Amblystoma* contained vesicles which stained blue when exposed to nile blue sulfate. Milaire (1959) found that in the mouse, crest cells could be distinguished from other cells by their high RNA content when fixed in FAA and stained with methyl green-pyronine. Di Virgilio *et al.* (1967) described crest cells in the chick based in part on their affinity for toluidine blue when fixed in 10% non-neutralized

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Fig. 1. Transverse sections through the caudal hindbrain of 7-somite mice. Scale bars represent 100 μm (A, C); 20 μm (B, D, E).

(A) Orientation section. Box defines (B).

(B) Note the light blue surface ectoderm (SE), dark blue neural crest cells (NC) emerging at or near junction (X) between surface ectoderm and intermediate blue neural plate (NP); also dark blue cell in neural plate (arrow in NP).

(C) Orientation section. Box defines (D).

(D) Note the relatively large intercellular spaces between crest cells – even those still largely within neuroepithelium (lower NC arrow; see also (B)). 'Ot P' marks the anterior edge of the otic placode, which contacts the basal surface of the neural plate thereby trapping (temporarily?) crest cells above.

(E) The tip of a neural fold showing a patch of neural crest cells. Some still maintain contact with the apical surface of the neuroepithelium, while others protrude from its basal surface.

Note other dark-staining cell types (e.g. endothelial cell at D(*)).
formalin. This stain appeared to be localized in concentrated cytoplasmic granules. Finally, Johnston & Listgarten (1972) have used an azure A staining technique for RNA's to help identify crest cells in the chick.

The present work describes a new histological technique which differentially stains the ectodermal epithelium of the neural folds. Among the most distinctive features resulting from use of this technique is a population of darkly stained cells thought to be in transition from ectodermal epithelial cells to crest mesenchyme cells. Taking advantage of this distinction, the technique is used to describe neural crest formation in the head of the mouse embryo. Some features of this process include crest cell formation from presumptive surface ectoderm, from a groove in the forebrain–midbrain fold and from the margins of the forebrain adjacent to the optic pit.

MATERIALS AND METHODS

Embryos were removed from female ICR/DUB mice (Flow Laboratories, Dublin, VA) at between 9 and 9.5 days of pregnancy (plug day = day 1). All extraembryonic membranes were removed and the embryos staged according to the number of somites present. Embryos were then fixed for 3 h in cacodylate-buffered (0.1 M; pH 7.3) modified Karnovsky's fixative (2% glutaraldehyde, 2% paraformaldehyde plus 0.25 ml 1% CaCl₂) with or without 0.5% cetyl pyridinium chloride (CPC; Sigma, St Louis) added. The tissue was postfixed in 0.1 M cacodylate-buffered 1% OsO₄, dehydrated through alcohols to propylene oxide and embedded in Araldite 502. Blocks were cut at 1 μm using a Porter Blum MT-2B ultramicrotome. The resulting sections were stained for 5-55 min in a 1% toluidine blue/1% sodium borate solution at 54–56 °C, covered and observed in the light microscope. The CPC in the fixative is required to obtain the staining pattern described in the Results. In its absence no differential staining is observed and the time required to effect an adequate stain is greatly increased. Differential staining appears to be a property of individual cells and not that of their position within the embryo. In isolated fragments of head fold, where crest mesenchyme is directly exposed to fixative, rather than via the surface ectoderm, crest stains as it would in the intact embryo. Pene-
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Neural crest formation in the head of the mouse embryo is, however, slow. If the fixation time is inadequate the deeper tissues of the embryo will appear faded.

The use of this technique to identify neural crest cells is limited to the time during and shortly after their formation. With the onset of crest migration, crest cells are quickly lost among cells of mesodermal origin (e.g. mesenchyme, endothelial cells and red blood cells; Fig. 1D) which stain with an intensity equal to that of the crest. In addition, while neural crest formation may continue up to and after closure of the neural tube, the distinction between the crest and neuroepithelium is gradually lost as the overall staining intensity of the neuroepithelium increases to match that of the crest (Fig. 13B). The interpretation of the dynamics of neural crest formation is aided by the fact that crest formation in any one embryo presents not just a single stage, but a sequence of stages along the embryonic axis (Weston & Butler, 1966; Tosney, 1978). In a given embryo, the most advanced stage is generally found in the midbrain/anterior hindbrain, with progressively earlier stages both rostral and caudal to that point. This pattern allows serial sections within the same region of the brain to be read as a time sequence. Throughout this paper, the different regions of the neural plate will be referred to according to the structures in the adult brain to which they will give rise. Such regions are identified using the neuromeres as described from the rat neural plate by Adelmann (1925).

For scanning electron microscopy, embryos were fixed in fixative without CPC, postfixed in OsO₄ and critical-point dried from 100% ethanol (Samdri, Tousimis, Rockville, MD.). These were then glued to studs using silver dope, sputter coated (Hummer V, Technics, Alexandria, VA) with gold-palladium and observed in a JEOL JSM-35C scanning electron microscope at 20 kV accelerating voltage.

RESULTS

Applying the technique described above, surface ectoderm stains light blue, neural plate an intermediate blue and cells thought to be neural crest, dark blue (Fig. 1A–E; see Fig. 10 for plane of section). The mechanism behind this differential staining is unknown. Therefore, the basis for identifying the dark blue cells as crest is the correlation between the timing and appearance of those cells and what is already known about the neural crest from previous

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Fig. 4. Scanning electron micrograph of a 5-somite mouse. Box in (A) defines (B). Note the neural folds approaching each other in the cervical region (small arrow, A); optic pits (large arrows) in the forebrain (FB). Dotted line shows approximate plane of section in Fig. 5. Scale bar represents 200 μm.

Fig. 5. Transverse section through the midbrain of a mouse at 5 to 6 somites. Scale bars represent 100 μm (A); 20 μm (B). (A) Orientation section. Box defines (B). (B) Note the notch (X), with a ridge ventral to it. Dark blue neural crest cells have delaminated from light blue surface ectoderm cells in the ridge region.
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histological and experimental studies. The following additional criteria both confirm this identification and further distinguish the crest from other cell types of the ectodermal epithelium:

(1) The ragged, broken contour of their basal surface. Frequently, a basal surface cannot be identified as crest cells cross it to escape from the epithelium. This criterion will distinguish crest cells from occasional dark staining cells otherwise clearly a part of the neuroepithelium (Fig. 1B). The exception occurs at the onset of crest formation when dark blue cells pause before escaping, and in so doing maintain the smooth, even contour of the basal surface of the epithelium (Fig. 5B; see below).

(2) The frequent presence of enlarged extracellular spaces around crest cells. These are clearest in embryos of more than five somites where cells of the neural ectoderm are otherwise closely packed. Such spaces may occur even around crest cells still at least partially within the epithelium (Figs. 1B, D). In the following, these criteria were used to provide a chronological description of crest formation in the head of the mouse.

The onset of crest formation

The first indications of crest formation may be seen in the midbrain and/or rostral hindbrain (i.e. rostral to the pre-otic sulcus) of the 3- to 4-somite embryo. At that age the neural plate is wide open and flexure of the plate has begun bending the forebrain over the rostral end of the embryo (Fig. 2). In such embryos, dark blue crest cells accumulate in the basal surface of the ectodermal epithelium in that region where the junction between the surface ectoderm and neural plate will soon form (Fig. 3A, B). These cells, not immediately released, maintain the smooth, even contour of the basal surface of the epithelium. The actual release of these cells as crest mesenchyme begins at 4–6 somites. The overall appearance of the embryo is similar to that at 3–4

Fig. 6. Scanning electron micrograph of a 5-somite mouse (same embryo as Fig. 4). Box in (A) defines (B). Note the groove in forebrain–midbrain fold (small arrow). Large arrows (B) mark the midbrain groove and beneath this groove a low ridge (R). Dotted line (B) shows approximate plane of section of Fig. 8. Scale bar represents 200 μm.

Fig. 7. Scanning electron micrograph of a mouse at 4 to 4+ somites. The embryo has been flipped on its back and we are looking up into fold between (under) forebrain and midbrain. Note the arrow in groove, and continuity of the groove with the midbrain groove (dotted line). Spur of the dotted line suggests future forebrain groove. Scale bar represents 50 μm.

Fig. 8. Parasagittal section through midbrain and forebrain region of a mouse at 3+ to 4 somites. Scale bars represent 100 μm (A); 20 μm (B). (A) Orientation section: box defines (B). (B) Note the forebrain–midbrain groove (arrow) with dark blue cells apparently escaping from the epithelium.
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somites except that the neural folds are approaching one another in the cervical region, and optic pits may be seen in the rostral surface of the now enlarged forebrain (Fig. 4). In such embryos, crest cells, which have accumulated in the basal surface of the epithelium, now delaminate from the light blue cells at the apical surface to escape as mesenchyme. The remaining apical cells then form surface ectoderm (Fig. 5A, B). This process results in the clear distinction between surface ectoderm and neural plate. Subsequently, crest cells form exclusively from the neural plate. Initially, crest cells continue to pause along the basal surface of the neuroepithelium before finally making their escape. Eventually, however, the crest cells no longer pause, and instead disrupt the basal surface of the epithelium while stretching from its apical to its basal surface (Fig. 1E). Crest cells, as they escape from the neural plate, are localized in the region at or near the junction between surface ectoderm and neural plate. This suggests that crest cells are released in a lateral to medial sequence with respect to the original neural plate. Towards the end of the 4- to 6-somite period, crest formation is also observed for varying distances into the caudal hindbrain (i.e. caudal to the pre-otic sulcus). There, the acute angle formed between the surface ectoderm and neural plate obscures details of the onset of crest formation. A less extensive version of the same process may occur.

Scanning electron micrographs of the midbrain/rostral hindbrain at 4–6 somites show large flattened cells of the surface ectoderm and small cobblestone-like cells of the neural plate (Figs. 4B, 9B) segregated from one another. At the line where the two cell types meet, a groove is formed, and just ventral to the groove, a low ridge (Fig. 6). In cross section, the counterpart of the groove is the notch formed at the junction between the surface ectoderm and neural plate, while the ridge corresponds to the region where the earliest crest is escaping, or has escaped from the surface ectoderm (Fig. 5B). At 4–6 somites the distinction between surface ectoderm and neural plate may be made from the midbrain back into the caudal hindbrain, but the groove is clearest in the midbrain/rostral hindbrain. No such distinction or groove can be found, as yet, in the forebrain.

Fig. 9. Scanning electron micrograph of a 6-somite mouse. Box in (A) defines (B). 'X' marks the site of the earlier forebrain–midbrain groove. Arrows indicate junction between surface ectoderm and forebrain neural plate. Scale bar represents 200 μm.

Fig. 10. Scanning electron micrograph of a 7-somite mouse. Long arrow suggests hook shape of the head of the embryo at this stage. The dotted line to the left shows approximate plane of section of Fig. 11. The dotted line to the right shows approximate plane of section of Fig. 1. Short arrow indicates pre-otic sulcus. Forebrain (FB) and hindbrain (HB) markers are on surface ectoderm covering those regions of the neural plate. Scale bar represents 100 μm.
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Crest formation from the forebrain–midbrain groove

In addition to the crest formation associated with the lateral margins of the neural plate, mesenchyme is formed in an unusual location at between four and five somites. When the forebrain bends with respect to the midbrain over the rostral end of the embryo, a fold is formed between the two (Figs. 6A, B). In the lateral portion of this fold, as it crosses beneath the rostral end of the midbrain ridge, a deep groove is formed which appears continuous with the groove along the lateral margin of the midbrain/rostral hindbrain (Fig. 7). Mesenchyme formation from the dark blue epithelium lining this groove is best seen in parasagittal sections (Figs. 8A, B). The formation of mesenchyme at this site is brief. The groove is gone, and has been replaced by evenly contoured surface ectoderm by 5–7 somites (Fig. 9). In parasagittal sections the dark blue cells lining the groove have been replaced by light blue surface ectoderm. The timing and location of mesenchyme formation from this groove suggests that it may be akin to that formed from the midbrain/rostral hindbrain ridge. However, here the entire ectoderm of the groove appears dark blue with no apical ectoderm apparent.

Crest formation in the forebrain

By 5–7 somites the neural folds have begun to fuse in the caudal hindbrain and midbrain flexure has tucked the forebrain under the rostral end of the embryo. As a result, the head of the embryo assumes a hook shape when viewed from the side (Fig. 10). This makes it possible to cut a single transverse section and see the midbrain plate on the dorsal surface and the forebrain plate with its invaginating optic pits on the ventral surface. Such sections reveal crest formation from the margins of the forebrain adjacent to the optic pits (Figs. 11A, B). The amount of forebrain crest formed appears to be greatest along its lateral margins. However, an occasional crest cell may be formed at the rostral margin of the forebrain as well (Figs. 12A, B). In scanning electron micrographs neural plate may now be distinguished from surface ectoderm, with a clear-cut boundary between the two (Fig. 9). The period from 5 to 7 somites also finds crest formation spreading the length of the hindbrain (Figs. 1A–E).

Fig. 11. Transverse sections through the forebrain and midbrain of a 6-somite mouse. Scale bars represent 100 μm (A); 20 μm (B). (A) Orientation section. Box defines B. (B) Neural crest being formed in the forebrain region, lateral to the optic pit (OpP).

Fig. 12. Parasagittal section through the forebrain of a 5-somite mouse. The plane of section is medial to that indicated in Fig. 6b. Scale bars represent 100 μm (A); 20 μm (B). (A) Orientation section. Box defines B. (B) Neural crest cells emerging from the rostral edge of the neural plate.
Fig. 13. Transverse section through a plane similar to that in Fig. 11, but in an 11-somite mouse. Scale bars represent 100 μm (A); 20 μm (B). (A) Orientation section. Box defines (B). Op V labels optic vesicle. A1 labels first pharyngeal arch. (B) Neural crest forming in the midbrain/rostral hindbrain (NP) at the time of neural tube closure.

Crest formation during and after fusion of the neural folds

Crest formation in the forebrain appears to be substantially complete by 7-10 somites. However, crest formation continues in the midbrain and hindbrain even after fusion of the neural folds to form a neural tube at between 8 and 16 somites (Figs. 13 A, B). This statement is based largely on the presence of cells which appear to be streaming from the neuroepithelium since the difference in staining intensity between the neural crest and neuroepithelium is largely lost by these stages.

DISCUSSION

Early crest migration in the forebrain

Crest cells formed at the margins of the forebrain appear to migrate dorsally over and around the optic pit. Some may remain there while others probably continue their dorsal migration until they are met by crest cells migrating ventrally from the midbrain. Those that remain might contribute to any of several structures in, or associated with, the eye (Johnston et al. 1979). Crest formed in the forebrain-midbrain groove of 4- to 5-somite embryos appears to gather beneath and just caudal to the optic pit, but may be excluded from this region as the pit enlarges to fill the space between itself and the surface ectoderm. These cells would then be forced caudally into the region of the first aortic arch. In any event, the pattern of crest migration in the forebrain and midbrain regions must be quite different from any identified in the chick.
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where the sites of crest formation, and its timing with respect to flexure and closing of the neural tube are likewise different.

The epithelium-to-mesenchyme transition

While the staining technique is clearly useful in looking at the time and location of crest formation, it also affords the opportunity to look at the earliest stages in the conversion of an epithelial cell into a mesenchyme cell. Specifically, the ability to identify early crest formation should be useful in studying the role of extracellular matrix in the transition from a neuroepithelial cell to a crest mesenchyme cell. Crest formation clearly involves the disruption of the basal lamina beneath the crest-forming epithelium (Meade & Norr, 1977; Tosney, 1978). In the present study, the appearance of the dark staining cells and disruption of the basal surface (basal lamina?) appear to be two separate events since during earliest crest formation, dark staining cells first accumulate in the basal surface of the epithelium without disrupting it (Figs. 3, 5).

Possible distinctions among crest cells

It is not known whether all neural crest cells are created equal. Much evidence has suggested that they are (LeDouarin & Teillet, 1974; Noden, 1975), but there remains evidence to the contrary (Cohen, 1977). It might, therefore, be asked whether mesenchyme formed from surface ectoderm is identical to that formed from the neural plate. In addition, crest cells from the neural plate appear to form from cells of progressively more medial origin. Are the early crest cells of more lateral origin equivalent to the later more medially derived cells? If not, perhaps the varying environment of the neural plate should be taken into account as a possible influence on the crest cells derived from it. That the properties of neural plate cells vary with time is suggested by the fact that early crest is derived from intermediate-blue-staining plate cells while later crest is derived from dark-blue-staining plate cells.

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