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Calcium and neurulation in mammalian embryos

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SUMMARY

The role of calcium in neurulation in rat embryos has been studied. Rat embryos at 10·4 days of gestation, when the cephalic neural folds have elevated but not fused, have been cultured in various media, and the effects of these media on the morphology of the cephalic neural folds have been observed by scanning and transmission electron microscopy. Embryos cultured in serum containing EDTA or EGTA, or in saline without divalent cations exhibit opening, then folding back ('collapse') of the cephalic neural folds. The neural cells lose their elongated shape and become rounded. Older embryos in which the cephalic neural folds have already fused do not show collapse of the neural tube. Culture of 10·4-day rat embryos with elevated but unfused cephalic neural folds in calcium- and magnesium-free saline to which either calcium or magnesium has been restored shows that calcium is the divalent cation which is essential for the maintenance of the elevated neural folds. In the presence of calcium, lanthanum, which competes for calcium sites, causes opening but not collapse of the elevated cephalic neural folds. Embryos treated with trypsin show dissociation of the lateral (non-neural) ectoderm but the neural folds remain elevated. If embryos in which the cephalic neural folds have been caused to collapse are further cultured in serum the folds re-elevate, although normal neural tube morphology is not completely regained. The possible implications of these observations to the understanding of the cellular mechanisms of normal neurulation, and of neural tube malformations are discussed.

INTRODUCTION

Neurulation is the morphogenetic movement by which the neural tube is formed from a flat plate of cells. The elevation of the neural folds, which are destined to fuse to form the neural tube, is accompanied by changes in the shapes of the cells of the neural ectoderm from polyhedral to elongated and tapered. Early experiments with avian and amphibian embryos in which the neural plate, isolated from the embryo, was shown to continue neurulation-like movements led to the idea that the cellular forces which effect neurulation are generated within the neural tissue itself (Roux, 1885; Glaser, 1914). More recently it has been suggested that active spreading of the lateral epidermis is one of the cellular forces which effect neurulation (Brun & Garson, 1983), but this explanation still requires that some contribution to neural tube formation is made by the cells of the neural tissue itself. Indeed computer simulations have shown that coordinated changes in the shapes of the cells of the neural tissue offer a sufficient explanation for the tissue

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movements seen during neurulation (Odell, Oster, Alberch & Burnside, 1982). Thus it seems reasonable to assume that neurulation is effected, at least in part, by co-ordinated changes in the shapes of the cells of the neural ectoderm (Karfunkel, 1974).

Microfilaments appear in the neural cells of amphibian embryos at the time when the cells are changing shape to become apically constricted (Schroeder, 1970; Burnside, 1971; Karfunkel, 1971; Perry, 1975). Furthermore, treatment of amphibian, avian or mammalian embryos with agents such as cytochalasins, which disrupt microfilament function, causes the neural folds to lose their apical constriction and the elevated neural folds to flatten (Karfunkel, 1974; Morriss-Kay, 1983). In addition, colchicine, which inhibits microtubule assembly and extension, causes flattening of the neural folds in mouse embryos (O'Shea, 1982). Thus it appears that the changes in cell shape which are involved in neurulation are generated at least partly by mechanisms effected by microfilament and microtubule action.

In non-embryonic systems the contraction of microfilaments is initiated by changes in the level of intracellular-free calcium (Hitchcock, 1977) and thus calcium is implicated as being important in morphogenetic movements such as neurulation (Stanisstreet & Jumah, 1983). Measurements of calcium in the medium in which amphibian embryos are developing have suggested that calcium is relocated during neurulation (Moran, 1976). In addition papaverine, which is thought to alter calcium fluxes (Imai & Takeda, 1967), can prevent neurulation in amphibian (Moran & Rice, 1970) and chick (Lee & Nagele, 1979) embryos, and its effect can be ameliorated or reversed by the addition of the divalent cation ionophore A23187, which increases the permeability of biological membranes to calcium (Reed & Lardy, 1972).

In mammalian embryos too the elevation and apposition of the neural folds is accompanied by changes in cell shape. The possible role of calcium in neurulation has been rather less studied in mammalian embryos, although observations on mouse embryos of the effects of cyclic nucleotides, which are thought to influence membrane permeability to calcium (Rasmussen, Goodman & Tenenhouse, 1972), support the idea that calcium is important in neurulation in mammalian embryos (O'Shea, 1981). In addition it has been shown recently that papaverine inhibits neurulation in mouse embryos (O'Shea, 1982). Of interest therefore was the observation made during a study of wound healing in rat embryos that chelation of divalent cations in the culture medium caused collapse of the elevated neural folds (Smedley & Stanisstreet, 1984).

The present experiments are designed to test more directly the importance of calcium in the morphogenesis of the neural tube in rat embryos by determining the effects on the elevated neural folds of removal of extracellular calcium or magnesium, and of the addition of lanthanum, which competes for calcium sites (Weiss, 1974). The results of some of these experiments have been reported in abstract form (Stanisstreet, Smedley & Moore, 1984).

MATERIALS AND METHODS

Embryo culture

Rat embryos at the neural fold stage were obtained from random-bred white Whistar rats at 10·4 days of gestation, timed from midnight preceding the morning on which vaginal plugs were observed (New, 1978). Embryos were dissected from the uterus in Hank's balanced saline containing $4{\cdot}2\times10^{-3}$ M-sodium bicarbonate (Flow Laboratories Ltd.). For most of the experiments embryos were used at the stage when the cephalic neural folds had elevated but not yet fused; for one series of experiments embryos were explanted 5 h later when the cephalic neural folds had fused. Embryos were cultured at 37 °C in rotating bottles according to the method of New, Coppola & Terry (1973). The cultures were equilibrated with a 20% O_2, 5% CO_2, 72% N_2 gas mixture to provide the oxygen tension appropriate for embryos of this stage (New, Coppola & Cockroft, 1976). Before culture the extraembryonic membranes of the embryos were opened with watchmakers' forceps to facilitate penetration of the culture medium. Embryos were cultured in a variety of media.

Preparation of media

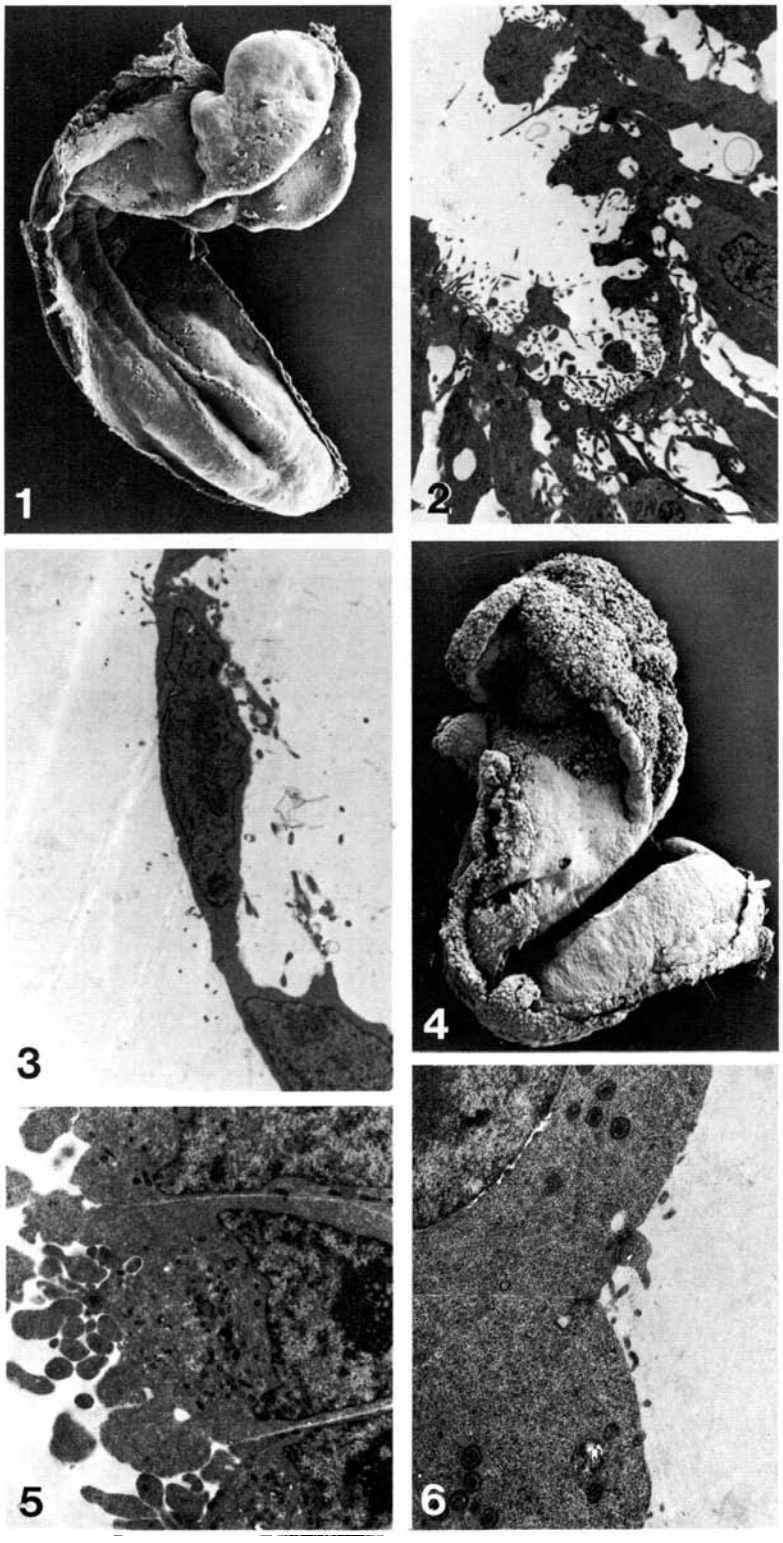
The rat serum was prepared as for long-term embryo culture (New, 1978). The blood was centrifuged immediately following withdrawal from the dorsal aorta and allowed to stand until the plasma clot had formed. The clot was removed and after recentrifugation the serum was collected. Streptomycin and penicillin (Flow Laboratories Ltd.) were added to final concentrations of 100 μg ml^{-1} and 100 i.u. ml^{-1} respectively and the serum was stored at −20 °C for a maximum of 28 days. Immediately before use the thawed serum was inactivated at 56 °C for 30 min. In some experiments either ethylene diaminetetra-acetic acid (EDTA) (B.D.H. Ltd.) or ethylene-glycol-bis (B-aminoethyl ether) N,N′-tetra-acetic acid (EGTA) (Sigma Ltd.) was added to a final concentration of $2{\cdot}5\times10^{-3}$ M.

Embryos were also cultured in Hank's saline with divalent cations or in calcium- and magnesium-free Hank's saline (both Flow Laboratories Ltd.). Unless otherwise stated the concentration of $NaHCO_3$ in the Hank's saline was supplemented to $1{\cdot}6\times10^{-2}$ M to provide a pH of 7·3 at 37 °C in equilibrium with 5% CO_2. In addition in the Hank's saline containing divalent cations the concentration of calcium was increased from $1{\cdot}3\times10^{-3}$ M to 5×10^{-3} M and the concentration of magnesium was increased from $8{\cdot}1\times10^{-4}$ M to 5×10^{-3} M to prevent a slight opening of the neural folds (see Results).

Addition of lanthanum chloride to the Hank's saline resulted in the formation of a precipitate since both lanthanum carbonate and lanthanum phosphate are insoluble. Therefore for experiments in which embryos were treated with lanthanum a modified saline was prepared without carbonate and phosphate ions, but with the concentrations of other ions increased to maintain the total ionic concentration. This saline comprised $1{\cdot}52\times10^{-1}$ M-NaCl, $5{\cdot}8\times10^{-3}$ M-KCl, 5×10^{-3} M-$CaCl_2$, 5×10^{-3} M-$MgCl_2$, $5{\cdot}6\times10^{-3}$ M-glucose, 2×10^{-2} M-Hepes buffer and 17 mg ml^{-1} phenol red. Embryos were cultured in this modified saline either alone (controls) or with 10^{-2} M-lanthanum.

Some experiments were conducted to determine the effect of cell dissociation on the elevated neural folds. It was noted that cell dissociation resulted if the pH of the medium was significantly different from 7·3. Thus for these experiments embryos were cultured in Hank's saline containing divalent cations but with either $4{\cdot}2\times10^{-3}$ M or 10^{-1} M-$NaHCO_3$ to provide pH values of 6·85 and 8·50 respectively at 37 °C in equilibrium with 5% CO_2. In addition some embryos were cultured in Hank's saline containing 20 mg ml^{-1} bovine pancreas trypsin (B.D.H. Ltd.) plus 10^{-1} M-$NaHCO_3$ to provide the optimum pH for trypsin activity.

With the exception of the series of experiments to determine the time course of the collapse of the neural folds embryos were cultured in the various media for 60 min. At the end of the culture embryos were observed by light microscopy and the state of the cephalic neural folds (elevated, open or collapsed) was noted. Embryos were then fixed for scanning or transmission electron microscopy.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Scanning electron microscopy

Embryos were fixed overnight in 2·5% glutaraldehyde in 10^{-1} M-cacodylate buffer, pH 7·3 (Karnovsky, 1965). They were washed in buffer and the extraembryonic membranes were removed using watchmakers' forceps. The embryos were dehydrated in a graded ethanol series, the absolute ethanol was replaced with liquid CO_2 and the embryos were dried by the critical-point method. The embryos were affixed to stubs, coated with gold–palladium and observed and photographed using a Phillips 501B scanning electron microscope.

Transmission electron microscopy

Embryos were fixed for 3 h in 1% osmium tetroxide plus 2·5% glutaraldehyde in 10^{-1} M-cacodylate buffer, pH 7·3. They were rinsed three times in buffer and the embryonic membranes were removed as above. The embryos were dehydrated in a graded ethanol series and embedded in Spurr's resin. Blocks were sectioned on a Reichert OM U3 ultramicrotome to produce 1 μm and ultrathin sections. The 1 μm sections were stained for 1 min with 1% toluidine blue and observed microscopically. The ultrathin sections were collected onto copper grids and were then double stained with uranyl acetate and lead citrate. The sections were observed and photographed using a Zeiss EM10C/CR transmission electron microscope.

With the exception of the lanthanum experiments, for each condition at least 20 embryos from three or more litters were observed by scanning electron microscopy. For the lanthanum experiments at least 10 embryos from two litters were used. All embryos within each experimental group reacted in the same way unless otherwise stated in the Results section.

RESULTS

The first series of experiments was conducted to determine the effects of the removal of divalent cations from the serum in which rat embryos at the elevated neural fold stage were cultured, and to compare the effects with those on embryos in which the neural folds had already fused. Initially embryos at 10·4 days of gestation were used (Fig. 1). Transmission electron microscopy of such embryos, fixed immediately following removal from the uterus showed that the surfaces of

Fig. 1. Scanning electron micrograph of 10·4-day rat embryo, cephalic neural folds are elevated but not fused. (×63)

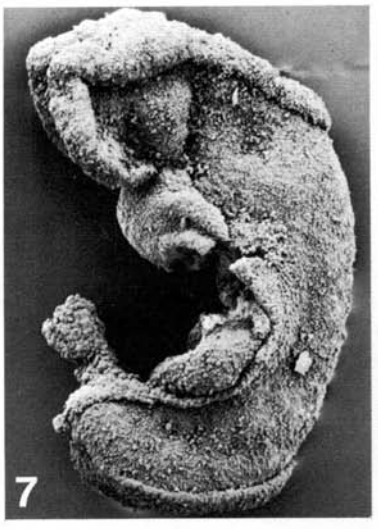
Fig. 2. Transmission electron micrograph of section through cephalic neural fold of 10·4-day rat embryo, in which cephalic neural folds have elevated but not fused. Neuroepithelial cells show convoluted surface with many projections. (×2350)

Fig. 3. Transmission electron micrograph of section through lateral ectoderm of 10·4-day rat embryo. Cells are flattened and show a smooth surface. (×3690)

Fig. 4. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in serum containing $2{\cdot}5\times10^{-3}$ M EDTA. Cephalic neural folds have collapsed. (×108)

Fig. 5. Transmission electron micrograph of section through cephalic neural fold of 10·4-day rat embryo, in which cephalic neural folds had elevated but not fused, following 60 min culture in calcium- and magnesium-free saline. Neuroepithelial cells show fewer surface projections than controls but still appear tightly joined.

Fig. 6. Transmission electron micrograph of section through lateral ectoderm of 10·4-day rat embryo following 60 min culture in calcium- and magnesium-free saline. Cells are more rounded than in controls but still appear tightly joined. (×11 660)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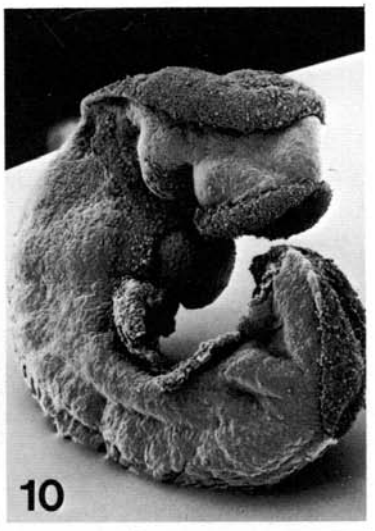
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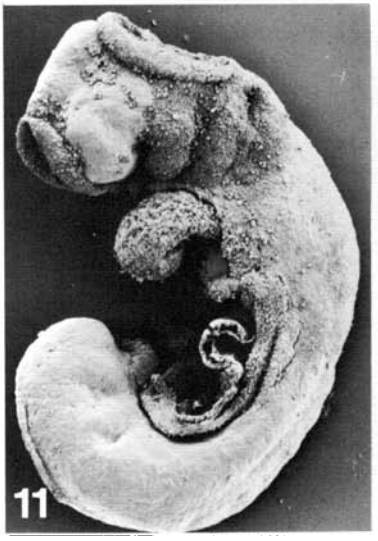
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the neuroepithelial cells were convoluted with many surface projections (Fig. 2). In contrast, cells of the lateral epithelium were flattened with a smooth surface (Fig. 3). Absence of divalent cations in the culture medium caused the neural folds to collapse; culture of embryos at the elevated neural fold stage for 60 min in either serum containing $2{\cdot}5\times10^{-3}$ M-EDTA or in calcium- and magnesium-free Hank's saline caused the neural folds to curl back (Fig. 4). Transmission electron microscopy showed that the cells of the neural epithelium had fewer surface projections (Fig. 5). Generally cells of the lateral ectoderm appeared similar to those of the controls (Fig. 6). Addition of the more specific calcium chelator, EGTA, to the medium at $2{\cdot}5\times10^{-3}$ M also caused collapse of the elevated neural folds (Fig. 7). In contrast when older embryos in which the neural folds had already fused were cultured for 60 min in either serum containing $2{\cdot}5\times10^{-3}$ M-EDTA or in calcium- and magnesium-free saline the neural tube did not reopen or collapse (Fig. 8). Thus removal of divalent cations reversed the elevation but not the fusion of the neural folds. For this reason embryos at the earlier, elevated neural fold stage were used in subsequent experiments.

The second series of experiments was conducted to determine whether the neural folds would remain elevated when embryos were cultured in Hank's saline rather than serum. In addition experiments were conducted to observe the time course of the collapse of the neural folds. Originally embryos were cultured in standard Hank's saline which contained $1{\cdot}3\times10^{-3}$ M-$CaCl_2$ and $8{\cdot}1\times10^{-3}$ M-$MgCl_2$. Such embryos showed a slight opening of the neural folds after 60 min in culture. Embryos cultured in Hank's saline with the $CaCl_2$ and $MgCl_2$ both supplemented to 5×10^{-3} M showed no opening of the neural folds after 60 min. Thus serum is not essential to the maintenance of the elevated neural folds. In

Fig. 7. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in serum containing $2{\cdot}5\times10^{-3}$ M-EGTA. Cephalic neural folds have collapsed. (×84).

Fig. 8. Scanning electron micrograph of 10·6-day rat embryo, in which the cephalic neural folds had fused, following 60 min culture in calcium- and magnesium-free Hank's saline. Neural tube has not opened or collapsed. (×42)

Fig. 9. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 15 min culture in calcium- and magnesium-free Hank's saline. Cephalic neural folds have opened. (×77)

Fig. 10. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 30 min culture in calcium- and magnesium-free Hank's saline. Cephalic neural folds have collapsed. (×73)

Fig. 11 Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in calcium- and magnesium-free Hank's saline. Cephalic neural folds have collapsed. (×72)

Fig. 12. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in calcium- and magnesium-free Hank's saline supplemented with 5×10^{-3} M-$MgCl_2$. Cephalic neural folds have collapsed. (×72)


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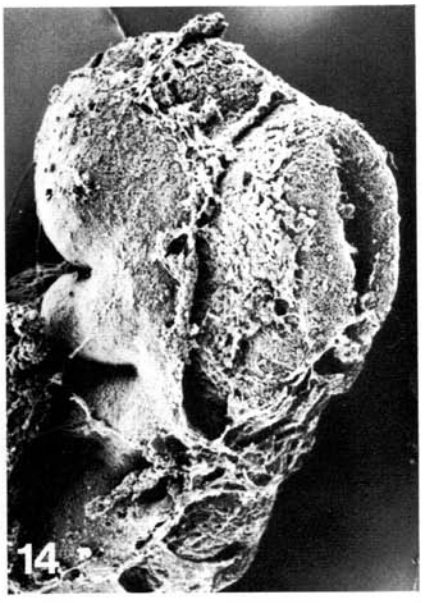
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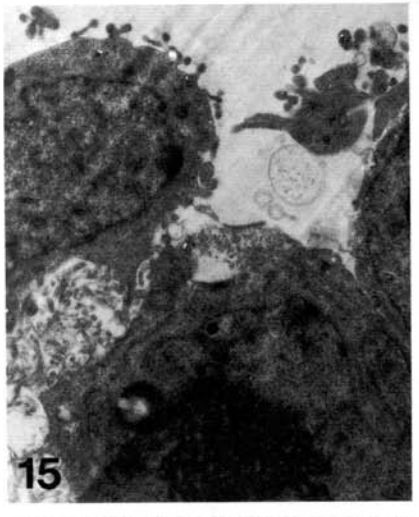
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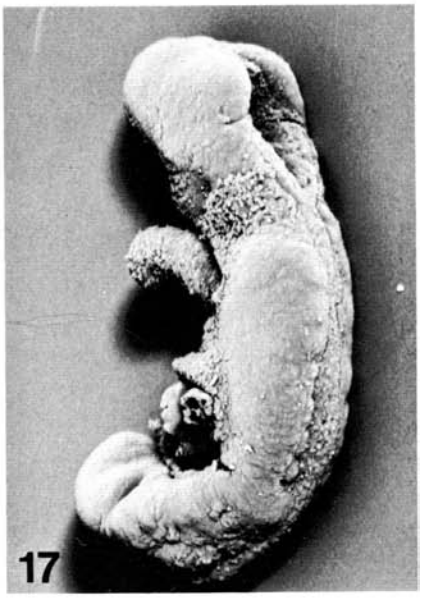
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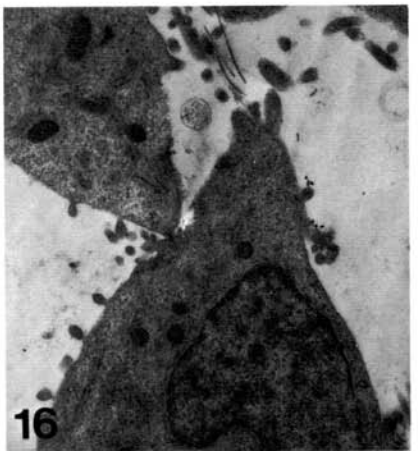
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subsequent experiments this modified Hank's saline with the divalent cations supplemented was employed. Embryos were also cultured for different times in calcium- and magnesium-free saline. After 15 min the cephalic and caudal neural folds had started to open in some of the embryos (Fig. 9); in the other embryos the cephalic and caudal neural folds had not only opened but had started to collapse. After 30 (Fig. 10) or 60 (Fig. 11) min the cephalic and caudal neural folds of all the embryos had collapsed, and the cells of the neural epithelium appeared rounded. Thus although serum is not essential to the maintenance of the elevation of the cephalic neural folds, calcium and/or magnesium are.

The results of the third series of experiments, designed to determine whether calcium or magnesium or both are required for the maintenance of the elevated neural folds, showed that in embryos exposed for 60 min to calcium- and magnesium-free saline supplemented with 5×10^{-3} M-$MgCl_2$ the neural folds had collapsed (Fig. 12). In contrast, embryos exposed for 60 min to calcium and magnesium-free saline supplemented with 5×10^{-3} M-$CaCl_2$ appeared normal; the neural folds remained elevated (Fig. 13). Thus it appears that calcium but not magnesium is essential for the maintenance of the elevated neural folds.

In the fourth series of experiments embryos were cultured in a modified saline (see Materials and Methods) with or without lanthanum chloride. Control embryos cultured in the modified saline, which contained divalent cations, appeared normal after 60 min; the neural folds remained elevated. Embryos cultured for 60 min in modified saline with divalent cations plus 10^{-2} M-$LaCl_3$ exhibited opening of the neural folds. Thus lanthanum causes the neural folds to open, even in the presence of calcium. Scanning electron microscopy revealed that

Fig. 13. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in calcium- and magnesium-free Hank's saline supplemented with 5×10^{-3} M-$CaCl_2$. Cephalic neural folds remain elevated. (×69)

Fig. 14. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in Hank's saline containing 20 mg ml^{-1} trypsin plus 10^{-1} M-$NaHCO_3$ to provide a pH of 8·5. Cells are dissociating but neural folds remain elevated. (×125)

Fig. 15. Transmission electron micrograph of section through cephalic neural fold of 10·4-day rat embryo, in which cephalic neural folds had elevated but not fused, following 60 min culture in Hank's saline containing 20 mg ml^{-1} trypsin plus 10^{-1} M-$NaHCO_3$ to provide a pH of 8·5. Cells are more rounded than in controls. (×5340)

Fig. 16. Transmission electron micrograph of section through lateral ectoderm of 10·4-day rat embryo following 60 min culture in Hank's saline containing 20 mg ml^{-1} trypsin plus 10^{-1} M-$NaHCO_3$ to provide a pH of 8·5. Cells are more rounded than in controls and cell junctions are becoming lost. (×8490)

Fig. 17. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, after 60 min culture in calcium- and magnesium-free Hank's saline followed by 120 min culture in rat serum. Cephalic neural folds, which had collapsed, have re-elevated, but brain vesicles are abnormal. (×66)

embryos cultured in lanthanum were covered with a layer of amorphous material, similar to the cell surface material observed when amphibian embryos are cultured in saline containing lanthanum (Rice & Moran, 1977), and possibly due to the reaction of the lanthanum with residual phosphate.

In the fifth series of experiments an attempt was made to deliberately dissociate embryos to determine whether the collapse of the neural folds was due to cell dissociation. Embryos were cultured for 60 min in Hank's saline with divalent cations but with the concentration of $NaHCO_3$ left at $4{\cdot}2\times10^{-3}$ M or adjusted to 10^{-1} M to provide pH values of 6·85 or 8·50 respectively at 37 °C in equilibrium with 5% CO_2. Scanning electron microscopy of such embryos showed that the lateral ectoderm was dissociated, but the neural folds had not collapsed. Similarly scanning electron microscopy of embryos cultured for 60 min in Hank's saline with 10^{-1} M-$NaHCO_3$ plus 20 mg ml^{-1} trypsin showed severe dissociation, but the neural folds remained elevated (Fig. 14). Transmission electron microscopy of trypsin-treated embryos showed the cells of the neural ectoderm were more rounded than those of the controls (Fig. 15). Cells of the lateral ectoderm were also more rounded and cell junctions were becoming lost (Fig. 16). Thus it appears that alteration of the pH of the medium and/or trypsin treatment does not cause collapse of the neural folds.

The final series of experiments was conducted to determine whether the collapse of the neural folds was reversible. Embryos were first cultured in Hank's saline at normal pH but without divalent cations. Light microscopical observations confirmed that after 60 min the neural folds had collapsed. The embryos were then transferred to rat serum for 120 min. Scanning electron microscopical observation showed that the neural folds of these embryos had re-elevated, although the morphology of the brain vesicles was abnormal (Fig. 17). Thus it appears that restoration of extracellular calcium causes the collapsed neural folds to re-elevate, but that the embryo does not recover a completely normal morphology.

DISCUSSION

The present results show that when rat embryos are cultured in appropriate conditions the elevated neural folds can collapse. Once the anterior neural folds have fused they do not collapse under the same experimental conditions. That the unfused neural folds remain elevated in a simple saline demonstrates that the macromolecular components of serum, which is required for the successful culture of embryos *in vitro* (Gupta & Beck, 1983), are not required for the maintenance of the elevated neural folds, at least for short periods. However, the results of experiments in which embryos were cultured in serum containing the divalent cation chelator EDTA or in calcium- or magnesium-free saline showed that divalent cations are essential to the maintenance of the neural folds, and thus for successful neurulation. That the neural folds collapse when embryos are cultured in serum with EGTA, which has a preferential affinity for calcium ions, suggests that calcium is the divalent cation required for the maintenance of the neural folds.

This suggestion is confirmed by the results of the calcium- or magnesium-replacement experiments, since they demonstrated that calcium protects against collapse of the neural folds but magnesium does not. This idea is also supported by the results of experiments in which addition of lanthanum to the medium in the presence of calcium caused the neural folds to open. Presumably lanthanum, which competes for calcium sites (Weiss, 1974), displaced calcium, essential to the maintenance of cell shape, from the cells of the neural folds.

The demonstration that a morphogenetic event, neural fold elevation, can be reversed emphasizes that during normal development the folds are actively maintained until their fusion. One requirement for this appears to be the presence of extracellular calcium. The concentration of calcium in the amniotic fluid, which bathes the neural folds *in vivo* at this stage, is not known. Culture of embryos in Hank's saline with $1{\cdot}3\times10^{-3}$ M-calcium chloride causes the neural folds to open slightly. The concentration of calcium in immediately centrifuged rat serum, which allows normal neurulation *in vitro*, was found by atomic absorption spectroscopy to be approximately 3×10^{-3} M. Assuming that the amniotic fluid achieves ionic equilibrium with the culture medium in longer term embryo culture, the concentration of calcium required for neurulation therefore appears to be between $1{\cdot}3\times10^{-3}$ and 3×10^{-3} M. If calcium is restored to embryos in which the neural folds have collapsed the folds re-elevate within two hours. Thus experimentally induced re-elevation occurs more rapidly than the morphogenetic event which it mimics, the elevation of the neural folds during normal development. This may be because the re-elevated folds are smaller than their normal counterparts, but it is also possible that during normal development the elevation of the neural folds is constrained by other factors such as the rates of synthesis or assembly of the microtubules or microfilaments which are required for neurulation (Karfunkel, 1975).

Transmission electron microscopy confirmed that the collapse of the neural folds was accompanied by a change in the shape of the cells of the neuroepithelium from elongated to rounded, and by a concommitant loss of apical adhesions of the cells. The correlation between cell shape and overall tissue morphology is compatible with the idea that coordinated changes in cell shape contribute to neural tube formation (Baker & Schroeder, 1967).

Treatment of embryos with trypsin caused severe dissociation of the lateral (non-neural) ectoderm, but the neural folds remained elevated. There appear to be two mechanisms of cell-to-cell adhesion, a calcium-dependent mechanism and a mechanism which relies on intercellular protein ligands and which is independent of calcium (Magnai, Thomas & Steinberg, 1981; Thomas & Steinberg, 1981). Since dissociation of the cells of the neural ectoderm might be expected to result in an alteration of the shape of the neural folds, it may be that cells of different tissues of 10·5-day rat embryos possess different mechanisms of adhesion; those of the lateral ectoderm being trypsin-sensitive and those of the neural ectoderm being calcium-sensitive.

A common type of abnormality produced by experimental treatment of mammalian embryos with a variety of teratogens is that in which the neural folds

remain open. Similarly, anencephalic and spina bifida birth defects form a significant proportion of the congenital malformations observed in humans. The primary lesion responsible for such defects is usually envisaged as the failure of the fusion of the neural folds. The present demonstration that the elevated neural folds can collapse under certain conditions draws attention to the possibility that such defects could be caused by a failure of the apposition of the neural folds. The rapidity of the collapse of the neural folds also emphasizes that a relatively transitory teratogenic insult might be sufficient to produce a major morphological abnormality. It may be significant too that in the present experiments re-elevation of the neural folds did not completely regularize development; brain vesicles did not appear entirely normal in embryos to which calcium had been restored.

Perhaps because the effects of addition of substances to the culture serum can be more readily assessed than the effects of removal of specific components from the culture serum, teratogenic conditions *in utero* are frequently considered in terms of exposure of the embryo to a foreign substance or to an abnormally high concentration of a natural substance. But abnormal development may also be due to a deficiency of certain factors. For example rat embryos appear to require specific factors for normal growth since culture of rat embryos in heterologous (e.g. human) serum is normal only if the serum is supplemented with homologous (i.e. rat) serum (Gupta & Beck, 1983). This principle may also apply to human development since periconceptual vitamin supplementation of women at risk of giving birth to malformed infants appears to reduce the risk to the foetus (Smithells *et al.* 1981), suggesting that a deficiency of certain factors may predispose towards abnormal development. The present experiments indicate that a local deficiency of calcium, even for a period which is short relative to the overall period of organogenesis, could produce a neural tube defect. In addition the possibility exists that other teratogenic agents which produce neural tube defects might act by affecting the calcium equilibrium of the neural cells.

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