Cellular contacts between hindbrain and prospective ear during inductive interaction in the axolotl embryo

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SUMMARY

In the amphibian embryo, beginning in the late neurula and continuing through midtailbud stages, the developing medulla exerts an inductive influence on the prospective ear, effecting its determination. Fine structural analysis of the region of closest apposition between the two tissues in the axolotl (Ambystoma mexicanum) reveals that during this period, there is a significant increase in the surface area of the apposed cells through the projection of long finger-like processes that traverse the medulla–ear interspace and the appearance of many focal contacts between the two cell types. These contacts are small, varying in diameter from 10-30 nm and they exhibit the septilaminar appearance and 2-4 nm intercellular cleft characteristic of gap junctions. Once the ear has been determined, both the cell processes and the focal junctions between apposed cells virtually disappear.

We suggest that the projection of processes from the surfaces of the apposed cells enhances the opportunity for cell interaction through the formation of very small gap junctions and that the junctions could provide the structural substrate for direct communication between medulla and ear during their inductive interaction.

INTRODUCTION

During amphibian gastrulation and early neurulation, the heart mesoderm and then the chordamesoderm exert inductive influences on the overlying prospective ear ectoderm to predispose it to form the ear, or vestibular apparatus (Yntema, 1933, 1950; Jacobson, 1963). Determination progresses as the neural tube forms and neural inductors come to lie beneath the mesoderm-primed ectoderm: thus hindbrain completes the induction of the ear (Stone, 1931; Harrison, 1936; Jacobson, 1963). The influence of the hindbrain (prospective medulla) is most effective late in neurulation and persists into mid-tailbud stages (Yntema, 1950; Jacobson, 1966). At the same time, the ear

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rudiment undergoes a series of complex morphogenetic changes that result in its transformation from a thickened ectodermal plate into a hollow sphere (Norris, 1892; Kaan, 1926).

Prior to medullary influence, the prospective ear ectoderm is equipotential in that any of its parts can give rise to any part of the ear depending upon its position in the whole. One aspect of the determination of the ear is its axial polarization, with the anteroposterior (a-p) axis being specified in the late neurula and the dorsoventral (d-v) axis later, in the early tailbud embryo (Harrison, 1936; Hall, 1939). The polarization of the ear rudiment can be interpreted to mean its compartmentalization, or loss of equipotentiality, and this loss can be attributed to the inductive action imposed upon it by the developing medulla.

The means by which the medulla transmits its instructive messages to the prospective ear are unknown, but there are several possible alternative mechanisms. Among them are interaction between macromolecules on the apposed surfaces of each group of cells; transmission of diffusible molecules from the inducing tissue across an interspace to the reacting one; and transmission of molecules through gap junctions between the cells of the interacting tissues. In our light-microscopic studies of the temporal sequence of development of the hindbrain in the Mexican axolotl, Ambystoma mexicanum, we noted extremely close apposition between the developing medulla and ear during the time in which the former is exerting an inductive influence on the latter. We were thus led to a fine structural analysis of the relationship between the brain and ear in embryos of the appropriate stages to see if there is a structural basis for communication between the two tissues.

**MATERIALS AND METHODS**

Fertilized eggs were obtained through natural matings between adults of the axolotl colony maintained in our laboratory. Embryos were kept in spring water at 11 or 17 °C until the appropriate stages were reached. Numerical designations of developmental age are based on Harrison’s (1969) staging of A. punctatum. To define the morphological relationship between the developing medulla and ear during the period of their inductive interaction (Harrison, 1936; Yntema, 1950; Jacobson, 1963), embryos from stage 19 (late neurula) through stage 29 (midtailbud) were prepared for light and electron microscopy.

Embryos were dissected free of surrounding jelly coat and capsule and held in Niou-Twitty’s complex salt solution (Flickinger, 1949) until immersed in 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein and 2% DMSO in 0.1 M cacodylate buffer, pH 7.4, at room temperature (after Kalt & Tandler, 1971). Following fixation for 12–16 h at 4–6 °C, the embryos were rinsed several times in cold 0.1 M cacodylate buffer (total time 30 min), postfixed for 2 h in cold 2% OsO₄ in cacodylate buffer, rinsed rapidly (1–3 min) in buffer and
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then in 30 and 50 % ethanol, stained en bloc for 1 h in 2 % uranyl acetate in 70 % ethanol, dehydrated in a graded series of ethanols, and embedded in Epon 812. The rapid passage of tissue through the buffer washes and alcohols resulted in improved retention of glycogen and other cellular components.

Serial cross-sections, 4 μm thick, were cut from the heads at ear level, mounted on glass slides, stained with toluidine blue and examined in the light microscope. The region of closest apposition between the developing medulla and ear was determined for each stage (as in Fig. 1) by comparison of successive sections. Subsequently, when such regions were localized in thick sections, thin sections were cut from the blocks, mounted on formvar-coated 100-mesh grids, stained with uranyl acetate and lead citrate, and photographed in a Philips EM 200 electron microscope. Two (and sometimes three) sets of thin sections, separated by 4–6 μm, were cut from three or more embryos of each developmental stage.

To identify the medulla–ear interspace with certainty in the electron microscope, single, very low-power survey micrographs were made of each thin section (see Fig. 6A). Then, with the interspace definitively localized, consecutive low-magnification micrographs (as in Fig. 6B) could be made along its entire length and high-magnification micrographs could be made of apposed medulla and ear cell surfaces (see Fig. 6C). Comparison of the low- and high-magnification micrographs with each other, and with the survey micrograph of the same section, allowed the unequivocal identification of tissue and cell types (as in Fig. 6).

RESULTS

In the late neurula, the neural folds are fusing to form the neural tube, the neural crest has not yet begun to migrate, and the prospective ear ectoderm has come into contact with the hindbrain (Fig. 1A). The ear rudiment is soon thereafter identifiable as the otic placode, a thickening of the inner or sensory layer of the ectoderm. The placode increases in thickness until early tailbud stages when it invaginates to form the otic cup (Fig. 1B). It then closes over to form the thick-walled otic vesicle and is no longer part of the ectoderm (Fig. 1C). During tailbud stages, the anterior and posterior borders of the ear rudiment are separated from the developing medulla by streams of migrating neural crest cells, whereas in between, no crest cells are interposed between the two tissues and the interspace is very narrow (Fig. 1B, C) and may not be detectable in the light microscope (as in Fig. 1B).

Fine structural analysis of the region of closest apposition between the developing medulla and ear revealed that during the period in which the medulla effects the determination of the ear rudiment, there is a significant increase in the surface area of the apposed cells through the projection of sometimes short but usually long finger-like processes that traverse the interspace (Figs. 2 and 3, respectively) and the appearance of many focal contacts between the two cell types. These contacts are small, varying in diameter from 10 to 30 nm (Figs. 2–4) and they exhibit the septilaminar appearance and 2–4 nm intercellular cleft
Fig. 1. Light micrographs of 4 μm-thick cross-sections through the heads of axolotl embryos at the level of closest apposition between the developing medulla (M) and ear. A labelled tracing of each section is on the right. e, Ectoderm; m, mesoderm; nc, neural crest; n, notochord. × 70. (A) Late neurula, stage 19. The prospective ear ectoderm (PEE) on the left is in contact with the medulla; on the right it is separated from the medulla by an artefactual space. The arrowhead indicates the site of neural fold fusion. (B) Early tailbud, stage 25. No interspace between the medulla and otic cup (OC) is apparent. (C) Midtailbud, stage 29. The area of contact between medulla and ear is more extensive on the right since the otic vesicle (OV) on that side has been sectioned through its center, whereas the other has not.
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(Fig. 4) characteristic of gap junctions (Decker & Friend, 1974). As Ginzberg & Gilula (1980) and Peng, Nakajima & Bridgman (1980) have observed, the membranes of the focal contacts show no particular specialization such as, for example, associated cytoplasmic density (Figs. 3 and 4).

In the late neurula (stages 19–22), processes from both medulla and ear cells extend into the interspace (Fig. 3), but their density is not as great as at later stages (see Fig. 10). In most instances, the identity of the cell giving rise to a particular process could be determined by comparison of adjacent sections, but occasionally the appropriate sections were not available and the cell of origin could not be established (Fig. 4). That such processes arise from either medulla or ear cells is certain since no other kinds of cells are present in the interspace of the region examined. Focal junctions unite prospective medulla and ear cells: they may connect a cell process with a cell body on the other side of the interspace (Fig. 3), a process with both medulla and ear cells (Fig. 4), a process with another process, or apposed cell bodies (Figs. 5A, 6). When the last is the case, the interspace narrows abruptly as the apposed cells approach each other to make multiple focal contacts (Fig. 6C).

By early tailbud stages 23–24, the sensory epithelium is thickening and the ear cells increase in number, changing their shape and orientation with respect to the interspace. This reorganization continues as the otic placode invaginates to form the otic cup at stages 25–27. During stages 23–27, the number of focal junctions and the length and density of the cell processes projecting into the interspace increase markedly, reaching a maximum at stages 24–25: at this time the medulla–ear interspace is difficult to define (Figs. 1B, 10A) because of the many processes contained within it (Fig. 10B). The interspace is so crowded that it is virtually impossible to determine from which cells the processes arise. As in the earlier stages, focal junctions are present between cell processes and cell bodies of the apposed tissue types (Fig. 2), between cell processes (Fig. 10B) as well as between cell bodies on opposite sides of the interspace (Figs. 5B, 7). In a single stage-23 embryo, short processes from medulla and ear cells crossed the interspace and at their points of contact, gap junctions were observed (Fig. 8). Gap junctions are common between adjacent cells within each of the apposed tissues (Fig. 9).

By midtailbud stages 28–29, ear cells facing the medulla are columnar and oriented perpendicular to the interspace. During these stages, the edges of the otic cup approach each other and fuse to form the otic vesicle. Crowding of the medulla–ear interspace with cell processes is greatly reduced so that it is narrow, relatively uniform in width and well defined, and there are few or no membrane contacts between apposed tissues (Fig. 11). Only an occasional focal junction is present, usually connecting a short cell process with a cell body on the other side of the interspace. Basal laminae, which were not apparent in the interspace in association with either medulla or ear cells at earlier stages (see Figs. 3, 4, 6 and 8), are present, albeit as discontinuous structures, by midtailbud stages.
DISCUSSION

In the amphibian embryo, beginning in the late neurula and continuing through midtailbud stages, the developing medulla exerts an inductive influence on the prospective ear, effecting its polarization. The a-p axis is established first, during late neurulation stages 19–22 (Harrison, 1936; Detwiler, 1940), and at that time, finger-like processes extend from both medulla and ear cells into the interspace between the interacting tissues and focal junctions connect the two cell types. Specification of the d-v axis begins later, in the early tailbud embryo at stage 23, and nears completion at stage 25 (Harrison, 1936; Hall, 1939) when the ear rudiment (otic cup) loses its equipotentiality and begins to compartmentalize (Kaan, 1926; Harrison, 1936). During this period, the length and density of cell processes projecting into the interspace as well as the number of focal junctions increase markedly. By midtailbud stages 28–29, only an occasional short cell process remains in the medulla–ear interspace and there are few or no focal junctions present between the apposed tissues. At this time the ear rudiment (otic vesicle) is considered determined (Kaan, 1926; Yntema, 1933; Harrison, 1936) and its compartmentalization accomplished so that the dorsal half of the otic vesicle will give rise to the endolymphatic sac and sensory areas of the saccule, and the ventral half, to the semicircular canals and utricle (Kaan, 1926; Harrison, 1936). The appearance of cell processes and focal membrane contacts between the developing medulla and prospective ear during the time in which the former is effecting the determination of the latter, and their virtual disappearance once determination has occurred leads to the suggestion of a role for these structures in the inductive interaction. Extension of long, interdigitating processes by medulla and ear cells would enhance the potential for interaction between apposed tissues through an increase in surface available for the

Fig. 2. Electron micrographs of the medulla–ear interspace in an early tailbud embryo, stage 23. A short process from a medullary (M) cell extends across the interspace to form focal junctions (arrows) with an ear (E) cell. × 52000. Inset: enlargement of the focal junctions in A. × 135000.

Fig. 3. Electron micrographs of the medulla–ear interspace in a late neurula, stage 19. (A) A long finger-like process from a medullary (M) cell traverses the interspace (arrows) to closely appose an ear (E) cell. × 8300. (B) Enlargement of the area within the rectangle in A reveals focal junctions (arrows) between the medullary (M) cell process and ear (E) cell body. × 52000. (C) Enlargement of the two lower focal junctions in B. × 135000.

Fig. 4. Electron micrographs of the medulla–ear interspace in a late neurula, stage 20. (A) A cell process (P) of unknown origin is located in the interspace, interposed between a medulla (M) and ear (E) cell. Focal junctions (arrows and double arrows) are present between the process and the medullary cell as well as between it and the ear cell. × 47500. Enlargements of focal junctions at double arrows in A between the process and a medullary (M) cell body is shown in B, and between the process and an ear (E) cell body, in C. × 135000.
formation of specialized membrane contacts. Since the observed focal junctions exhibit the septilaminar appearance and 2–4 nm intercellular cleft characteristic of gap junctions (Decker & Friend, 1974), it may be that they serve as communicating junctions, providing the structural substrate through which the medulla transmits its inductive messages to the ear rudiment.

There is a growing body of evidence that is consistent with the idea that gap junctions are, in fact, the structural basis for the electrotonic coupling of cells as well as for the direct intercellular transfer of molecules (see review by Bennett & Goodenough, 1978). Electrotonic coupling between embryonic cells appears to be widespread (Ito & Loewenstein, 1960; Sheridan, 1966, 1968; Bennett, Spira & Pappas, 1972; Hanna et al. 1980; among others) and it is possible that coupling functions in the intercellular communication known to be essential to embryogenesis (Potter, Furshpan & Lennox, 1966). Gap junctions are present in embryos (Lentz & Trinkaus, 1971; Bennett et al. 1972; Revel, Yip & Chang, 1973; Decker & Friend, 1974; Keeter, Pappas & Model, 1975; Hanna et al. 1980; Ne’eman, Spira & Bennett, 1980; among others). If gap junctions serve as pathways for the transfer of instructive molecules between cells during development, their disappearance would block such communication. Dixon & Cronly-Dillon (1972) have described transient gap junctions that link differentiating retinal ganglion cells in Xenopus laevis embryos at the time that specification for their projection onto the tectum is occurring. Just after specification, the junctions disappear.

Small membrane contacts that resemble tiny gap junctions in both thin section and freeze-fracture preparations have been observed to connect cells in adult tissues: for example, they are present between rod and cone photoreceptors in monkey (Raviola & Gilula, 1973) and toad retinas (Fain, Gold & Dowling, 1976). That such junctions (Raviola, 1976) may function in coupling is supported by the fact that in the cat at least, cone cells receive substantial electrical input from rods (Nelson, Kolb, Famiglietti & Gouras, 1976). Focal junctions are present in embryonic tissue as well. Decker & Friend (1974) describe small ‘gap-like’ junctions between cells of the prospective

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Fig. 5. Electron micrographs of focal junctions (arrows) between medulla (M) and ear (E) cell bodies from (A) a late neurula, stage 19, and (B), an early tailbud embryo, stage 23. The extracellular space is the interspace. × 68000.

Fig. 6. Electron micrographs of the prospective medulla and ear in a late neurula, stage 22. This series of micrographs illustrates our method of unequivocal identification of cell type. (A) A scan micrograph of an entire thin section, showing the developing medulla (M), the ear rudiment (E) or otic placode, and the overlying ectoderm (e). m, Mesoderm; arrows indicate the medulla–ear interspace. × 2300. (B) A low-magnification micrograph of the medulla (M)–ear (E) interspace (arrows) within the rectangle in A. 1, Lipid droplet; y, yolk platelet. × 9000. (C) A higher magnification micrograph of the medulla–ear interspace (I) within the rectangle in B showing focal junctions (arrows) between medulla (M) and ear (E) cell bodies. × 68000.
neuroectoderm in the amphibian late gastrula and early neurula as appearing in thin sections as septilaminar appositions with 2–4 nm intercellular clefts and in freeze-fracture replicas as small, usually amorphous aggregates of 4–30 closely packed 8–9 nm P face particles, with corresponding pits on the E face. These particle groups are interpreted to be nucleation sites for developing gap junctions, with the latter enlarging as its prospective particles merge with the differentiated particles at the periphery of the small aggregates. Ginzberg & Gilula (1980), on the other hand, suggest that since they have no later correlate, the gap-like focal junctions between afferent nerve endings and hair cells in the embryonic chick labyrinth may be transient gap junctions. Peng et al. (1980), having observed identical junctions connecting Xenopus nerve and muscle cells only in cultures less than 1-5 days old, also proposed that such junctions are transient structures. These two interpretations of the nature of the focal junctions present during embryogenesis are not mutually exclusive: the junctions may represent either developing gap junctions or transient ones, depending upon the kinds of cells that they unite. Trelstad, Hay & Revel (1967) suggested that gap junctions are at first very small, but that subsequently those between cells in the same tissue become more extensive, while those between cells in unlike tissues disappear.

Focal close contacts have been observed between cells of unlike tissues during their inductive interaction: for example, they are present in the amphibian gastrula, between cells of the archenteron roof and prospective neuroectoderm (Nakatsuji, 1975); in the chick gastrula, between cells of the mesoblast and epiblast (prospective neuroectoderm) (Trelstad, Revel & Hay, 1966; Trelstad et al. 1967); in the rat embryo, between apposed cells at the epithelial-mesenchymal interface of the developing submandibular gland (Cutler & Chaudhry, 1973) as well as of developing duodenum (Mathan, Hermos & Trier, 1972); and in the mouse embryo, between epithelial and mesenchymal cells in the developing kidney (Lehtonen, 1975). In each of these systems, cell processes often extend between the two tissues, bringing them into close apposition. Various functions have been ascribed to the focal contacts between interacting tissues: it has been suggested that they serve as labile points of adhesion between cells that are

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Fig. 7. Electron micrographs of the medulla–ear interspace (big arrows) in an early tailbud embryo, stage 23. Focal junctions (small arrows) are present between medulla (M) and ear (E) cell bodies. × 68000. Inset: enlargement of the focal junctions. × 135000.

Fig. 8. Electron micrographs of the medulla–ear interspace in an early tailbud embryo, stage 23. (A) A short process from an ear (E) cell extends across the interspace (arrows) to contact a medullary (M) cell. × 11000. (B) Enlargement of the area within the rectangle in A reveals that the contact between medulla (M) and ear (E) is a gap junction. I, Interspace. × 68000.

Fig. 9. Electron micrographs of gap junctions between (A) adjacent ear (E) cells and (B) adjacent medullary (M) cells in early tailbud embryos, stage 24. × 68000.
Fig. 10. Electron micrographs of the medulla-ear interspace in an early tailbud embryo, stage 25. (A) The interspace (arrows) is difficult to distinguish because of the presence of many processes from both medulla (M) and ear (E) cells. × 16500. (B) Enlargement of a portion of the interspace from a section adjacent to that shown in A. Numerous focal junctions (arrows and double arrow) are present between the cell processes and medulla (M) and/or ear (E) cell bodies as well as between the processes themselves. Vertical bars indicate the interspace. × 100000. (Inset) Enlargement of the focal junction at the double arrow in B. × 135000.

Fig. 11. Electron micrograph of the medulla-ear interspace in a midtailbud embryo, stage 29. The interspace (arrows) between the developing medulla (M) and ear (E) is well defined: no cell processes and no focal junctions are present between the apposed tissues. × 16500.
changing position with respect to one another (Nakatsuji, 1975) and further, that they have a role in contact inhibition of migration, perhaps by mediating coupling between cells (Trelstad et al. 1967), or that they serve (in some unspecified way) in the inductive interaction itself (Mathan et al. 1972; Cutler & Chaudhry, 1973), perhaps by making communication direct through cell surface or cell junction mediated interaction (Lehtonen, 1975, 1976; Saxen & Lehtonen, 1978). In any case, it is implicit in both interpretations that, irrespective of function, focal contacts between dissimilar embryonic tissues are likely to be transient in nature.

In light of all of the above, it is our view that the focal contacts that we observe between the developing medulla and ear during their inductive interaction are, in fact, very small gap junctions and as such, provide the structural substrate for direct communication between the two tissues. Once determination of the ear rudiment has been effected by the medulla, such communication is no longer needed and the junctions are lost. The focal junctions, although tiny, exhibit the morphological characteristics of gap junctions, and the single embryo in which ordinary gap junctions were observed to connect medulla and ear cells gives support to our belief that gap junctions can form between different interacting tissues in an induction system.

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