COMPARTMENTAL STRUCTURE OF THE OTIC PLACODE: JUNCTIONAL PERMEABILITY AND CLONAL RESTRICTION

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The *otic placode* is induced in the head ectoderm by signals which are believed to arise from the neighbouring mesoderm and the neural tube. Specification of the otic domain starts very early in development, but the otic primordium is fully committed at the placode stage, when the first evidence of a morphological structure associated with the ear is apparent. The purpose of this work was to study whether the otic placode forms a developmental *compartment*. This may be defined as structural and functional domain which is isolated from the surrounding and originally equivalent territory, and that is developmentally committed. Operationally, it can be identified by demonstrating boundaries and restricted communication between the proposed compartment and its surroundings. The present experiments were carried out in order to study: 1) the occurrence of limits to intercellular communication via *gap* junctions between otic and extra-otic ectoderm, 2) the presence of clonal restriction within the otic placode. The results show that the otic placode forms an uniform compartment which is limited by the *otic ridge*. This forms a functional boundary between the otic placode and the surrounding ectoderm regarding cell-cell communication and clonal expansion.

Experiments were carried out on chick embryos from stage 11 to 14 (Hamburger & Hamilton, 1951). For dye injection, embryos were dissected, the heart primordium removed and immobilised on a *Sylgard* coated well and viewed under a binocular microscope. Microinjection was carried out following Fraser *et al.*(1990) and Martínez *et al.*(1991). Pipettes were pulled from borosolicate glass, tips filled with the corresponding dye solution and backfilled with 1 M LiCl. *Biocytin* (Sigma, 30mg/ml) and *lysinated rhodamine dextran* (LRD) $10x10^3$ mw, Molecular Probes, 100mg/ml were micro-iontophoretically injected using a conventional microelectrode amplifier with 0.5-1 nA/500 ms current pulses for 2-3 min for biocytin injections or 30-45 sec for LRD. Electrode resistance was between 70 and 150 Mohm. Cells with membrane potentials lower than -15mV were discarded and those used for this work were typically about -40 mV. Specimens were fixed for two-three hours with 4% paraformaldehyde in 0.1 M phosphate buffer, after 20 min for biocytin injections. For clonal studies with LRD injections, the otic region was explanted and cultured for 36 hours on collagen gels with M-199/Hank´s medium and 5% fetal calf serum added. Explants were transversal sections of the embryo containing the neural tube and the otic placodes. Embryos were usually dissected further and flat mounted to be examined under epifluorescence microscopy and bright field illumination. *In situ* mRNA hybridisation was performed following the method described by Nieto *et al.*(1996) and *whole mount* immunodetection. The *cek*-8 probe was a kind gift of David Wilkinson and the *HNK-1/Leu-7* antibody was from Beckton and Dikinson.

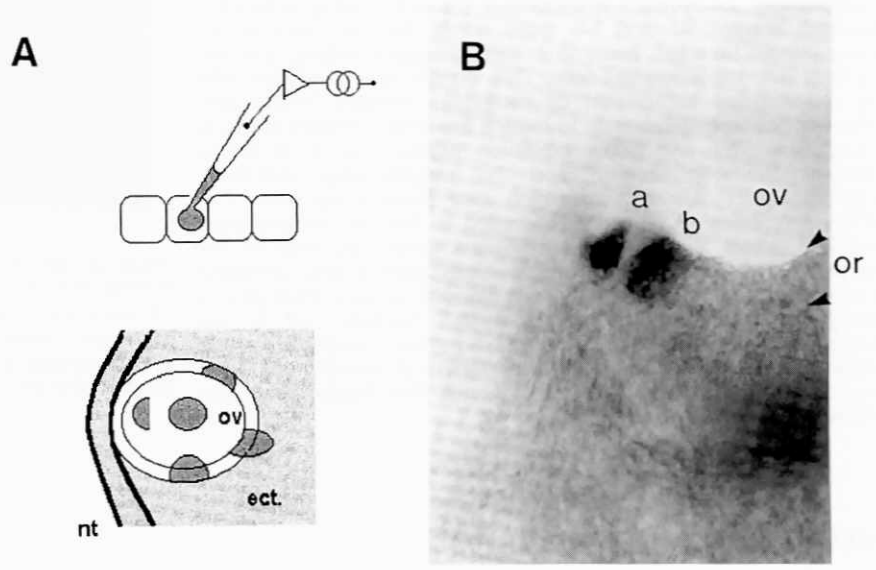

Figure 1. Restricted junctional permeability at the *otic ridge*. **A**, Squematic diagram of the experiment and the possibilities expected for the diffusion of biocytin into neighbouring cells. **B**, *Whole-mount* of a stage 13 chick embryo. Two cells located at the *otic ridge* , **a** and **b** were injected with biocytin for 3 and 1 minute, respectively. **ov**= otic vesicle, **or**= otic ridge. Exp.27296E12. Magnification=100x

Microinjections of *biocytin* were carried out on the edges and within the body of the otic placode. Injections performed at the bottom of the otic placode were typically oval or round shaped, expanded several cell diameters and showed a high dilution of the dye. In contrast, injections at the otic ridge showed sharp edges, expanded only few cell diameters and were intensely stained (Fig. 1). Diffusion of the dye out of the ridge was limited both towards the ectoderm and towards the placode, and also laterally within the axis of the ridge. Similar results were obtained using *Lucifer yellow* as a dye. We examined a total of eleven ridge injections and eighteen body injections. The internal aspect of the otic placode is in close apposition to the neural tube. Biocytin injections on cells at the neural tube or the otic placode, however, never expanded into each other territory.

Single-cell injections of LRD (n=10) were carried out following a similar procedure (Fig. 2). Injections at the bottom of the otic placode revealed clones of 6 to 8 cells that expanded through wide regions of the otic epithelium, where they became intercalated among neighbouring -unlabelled- cells. Cells injected on the otic ridge exhibited a lower mitotic activity, clones usually consisting of 3 cells. Cells at the ridge remained in clusters and did not move out of the ridge. They tended to divide never radially but along the axis of the perimeter of the otic vesicle. Therefore, cell division and mobility within a clone were high at the body of the otic placode or the otic pit but severely restricted at the otic ridge, which formed effectively a boundary for clonal mobility.

Experiments were also carried out to study the expression of *cek*-8 and HNK-1 were studied with *in situ* techniques. *cek*-8 expression was intense in rhombomeres 3 and 5 and the otic placode (see Nieto *et al.*, 1992). The *cek*-8 signal was asymmetrically distributed within the otic placode. It was restricted to the otic placode and did not invade the surrounding ectoderm. The boundary between *cek*-8 positive and *cek*-8 negative cells coincided with the otic ridge, but it was not possible to be completely defined at the boundaries. However, two cells out of the otic ridge showed ectodermal cells that were definitively negative to *cek*-8 expression. HNK-1 antigen was also regionalised within the otic placode, being more intense to the lateral and caudal regions. The boundary between the areas that were positive and negative to HNK-1 also coincided with the otic ridge.

The results show that the otic placode forms a developmental compartment, functionally isolated from the surrounding ectoderm. Between stages 10 and 14, cells *within* the otic placode do communicate between each other through gap junctions and mix together with neighbouring cells. This occurs simultaneously with the regionalized expression of molecular markers which may reflect or perhaps collaborate to induce the establishment of future boundaries. The *otic ridge*, however, appears as a boundary between the otic domain and the neighbouring ectoderm, regarding both cell communication and clone expansion. The affinity properties of the cells belonging to the otic compartment probably reflect the operation of genes which are high in the hierarchy of pattern specification. The *cek*-8 gene is one candidate to mediate cell interactions at the otic placode since it belongs to a family of tyrosine kinase receptors that mediate cell to cell recognition and are probably involved in pattern formation. Further experiments on the precise temporal pattern of formation of these boundaries and on the function of genes that display early otic expression domains are required.

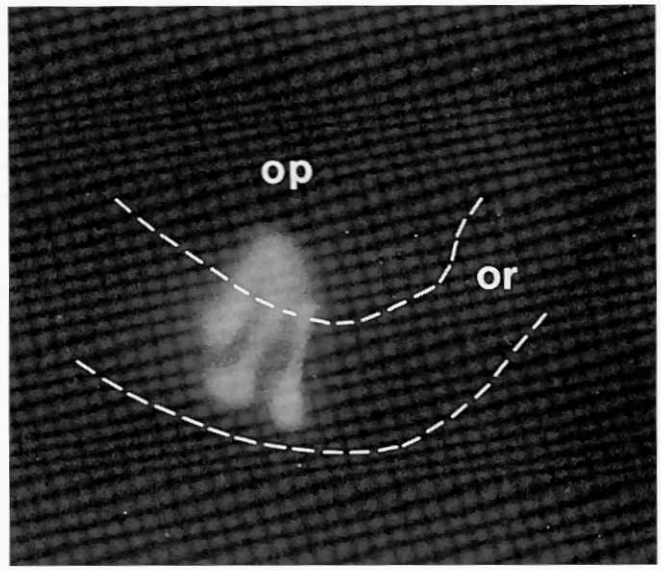

Figure 2. Cell clone at the *otic ridge*. A single cell was impaled and injected with LDR for 50 sec. It was cultured in a collagen matrix gel for 36 hours and examined under epifluorescence. Contours of the *otic ridge* are indicated by the traces and were drawn from the transilluminated image. **op**= otic placode. **Or**= otic ridge. Exp.20696E5. Magnification=200x

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