THE DETERMINATION OF THE AUDITORY PLACODE IN THE CHICK

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(With Six Text-figures)

INTRODUCTION AND METHODS

No direct experiments on the determination of the ear vesicle of chick or other avian embryos have been reported in the literature. Szepsenwol (1933) has described evidence derived from embryos with malformations of the head (omphalocephaly induced by electrolytic injuries to the unincubated blastoderm) which led him to suppose that the ear is induced by the acoustico-facialis ganglion, since he found no case in which the ear was present in the absence of this structure. Dalcq (1933) and Holtfreter (1933), working on amphibian embryos, have pointed out that in that group the otic placode is present before the acoustico-facialis ganglion is developed and can therefore scarcely be induced by it. It is difficult to be certain what constitutes the first appearance of the ear in the chick. In the four- to five-somite stage, just before the first cells leave the neural crest to form the acoustico-facialis ganglion, there is a thickened area of ectoderm in the region from which the ear will develop, but the thickened area is of much larger extent than the definitive ear placode and the cells are still vacuolarized like the rest of the epidermis. Moreover, this thickened area has at best a very incomplete determination, since isolated ectoderm from this region does not differentiate to an ear till a later stage, with about nine somites. We cannot, therefore, dismiss Szepsenwol's suggestion on the ground that the ganglion does not appear early enough.

The experiments to be described here were performed on chick embryos between the stages with three and twenty-one somites, and the operated embryos were subsequently cultivated *in vitro* for 1 or 2 days. The operations performed were as follows: (1) removal of the presumptive otic ectoderm and cultivation of it in isolation; (2) removal and cultivation of the part of the neural tube, including the presumptive neural crest, contiguous to the ear; (3) grafting of the neural tissue described above in contact with non-presumptive ear ectoderm; (4) removal of both the presumptive ear ectoderm and the neural tissue. The operations were usually made with glass needles, since this allows of more exact work, but the comparative toughness of the tissue made it advisable, after dissecting free a flap of tissue with glass, to effect its final removal with steel needles. In removing the ear ectoderm without the neural crest, or the neural crest without the ear ectoderm,

a cut was first made along the neural crest, and control specimens fixed immediately after the operation show that in fact the cuts made in this way did separate these two tissues quite cleanly, very little ectoderm being left adhering to the edge of the neural tube.

The exact position of the presumptive ear ectoderm, and the neighbouring neural plate, has never been described. Gräper (1930) has demonstrated a movement forwards of the neural plate relative to the somites in the early somite stages, so that one may expect that the presumptive ear region in embryos of three or four somites lies relatively farther posteriorly than does the otic placode at the time of its appearance. The operations reported here seem to support this suggestion; in some embryos in which the neural crest was removed back to the level of the first intersomitic groove, the wounded region in the developed embryo reaches posteriorly no farther than the middle of the otic placode. It is possible, however, that the wound had originally extended farther posteriorly but that after development its full extent is concealed by complete healing of its posterior part. In most of the operated embryos the defects were of larger extent, so that errors due to an incorrect estimation of the position of the presumptive tissues can hardly have arisen.

REMOVAL OF THE PRESUMPTIVE EAR ECTODERM

Thirty-one embryos from which the presumptive ear ectoderm was removed have been fixed and sectioned. The size of the piece of tissue removed varied somewhat in different experiments, but it was always large in comparison with the size of otic placode when it first appears. Usually the excision extended from the border of the anterior somite to the posterior edge of the midbrain and from the ridge of the neural fold well out into the somatopleure of the amnio-cardiac vesicle. The head mesenchyme was probably usually rather little injured in the operation but in the lateral regions the somatic mesoderm was removed adhering to the overlying ectoderm (Fig. 1).

Nearly all the operated embryos showed an ear on the side from which the presumptive ear ectoderm had been removed. This is true even of embryos operated as late as fifteen somites, by which time the otic placode is clearly recognizable in sections as a thickened patch of ectoderm. An embryo operated at twenty-one somites, however, showed no sign of any regeneration of the ear. The numbers available are not sufficient to fix precisely the latest time at which ear regeneration is possible, but it seems to be some time between the fifteen- and twenty-one-somite stages.

The ears formed in the operated embryos are usually smaller than the normal ear on the unoperated side of the embryo (Fig. 2); only in embryos operated as early as the three- to five-somite stages are they equal in size to the controls. The size of the regenerated ear seems in fact to be inversely proportional to the age of the embryo at the time of operation, but the difficulty of quantitatively standardizing the operations has made it impossible to investigate the relationship accurately.

The material from which the regenerated ears are formed presumably comes from the ectoderm surrounding the wound, which grows over and covers the excised

area. This repair of the wound is always complete. The region of the gill arches was also affected by the operation, and the repairing non-presumptive ectoderm is also capable of forming normal arches in the wound region, though the openings of the visceral pouches to the exterior tend to be larger than normal.

DIFFERENTIATION OF THE ISOLATED PRESUMPTIVE EAR ECTODERM

The capacity for differentiation of the presumptive ear ectoderm removed from the embryos described above was tested by placing the ectoderm in the amnio-cardiac vesicle, where it either lay free in the space or became attached to the host mesoderm in a region where it was unlikely that there would be any influences tending to affect its differentiation. The isolated fragments of ectoderm always rounded up into a little ball, which usually contained a certain amount of mesoderm, probably derived from the somatic mesoderm of the lateral part of the excised area. In fragments from embryos younger than about nine somites, no ear ever differentiated in the isolates. The ectoderm is everywhere highly vacuolarized, like the normal chick epidermis, and is mostly thin with occasional thickened invaginations which represent the gill arches. Small patches of ear tissue occur in the grafts from later stages, recognizable as darkly staining invaginated vesicles. They are usually small and it is impossible to say how far they represent a complete ear or only a part of one. Only in the case of the twenty-one-somite chick is the isolated ear quite normal and similar to the control.

REMOVAL OF THE NEURAL FOLD

In removing the neural fold, a cut was first made along the line of junction of the neural fold and the ectoderm, and the lateral wall of the fold was then cut along a longitudinal line about half-way between the neural crest and the floor of the groove. By freeing the two ends, a narrow strip of the upper part of the neural fold could thus be removed, extending from the level of the first somite to the midbrain region. The isolated pieces of neural tissue were inserted either into the amnio-cardiac vesicle or under the ectoderm of the more anterior parts of the head.

The operation should have removed the neural tissue which might be supposed to be the inducer of the ear vesicle, leaving the presumptive ear ectoderm untouched. As a matter of fact, a few of the isolated pieces of neural tissue contained small patches of adherent ectoderm, which have developed into tiny pouches of tissue similar to that of the otic placode. The injuries to the presumptive ear ectoderm are, however, trifling in extent.

One could only expect the operation to suppress the formation of the ear if (1) the ear ectoderm, at the time of the operation, was not capable of forming an ear when isolated, and (2) the neural tissue which was removed was the only tissue capable of endowing it with this capacity. Fifteen embryos are available in which the removal of neural tissue was made in stages earlier than the nine-somite stage; at this time, as we have seen above, the ear ectoderm is not able to develop into an ear vesicle when isolated. Of the fifteen embryos, eleven developed ear vesicles

which, though smaller than normal, were definite and apparently complete; two developed extremely reduced ears of the kind found when both the neural fold and the presumptive ear ectoderm is removed (see next section) and probably represent cases in which the operation was incorrectly carried out.

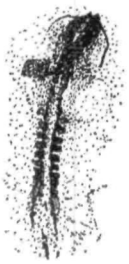

Fig. 1

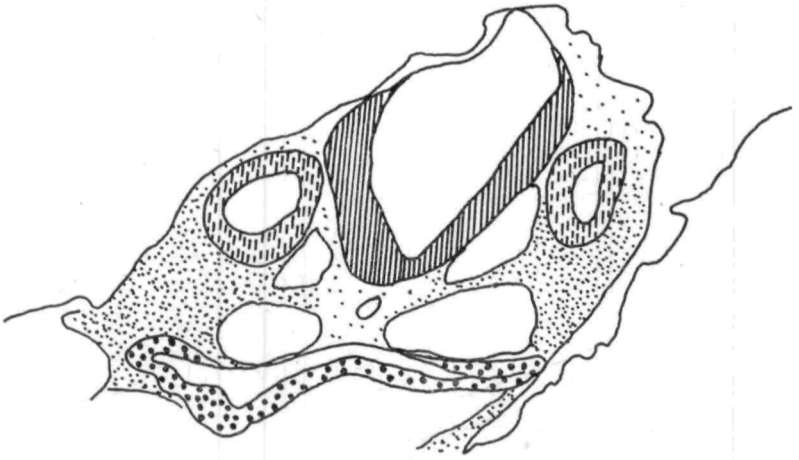

Fig. 2

Fig. 1. No. 35–0103. The right presumptive auditory ectoderm was removed and placed in the amnio-cardiac vesicle on the left side. View of the embryo fixed immediately after the operation (six somites).

Fig. 2. No. 35–073. Right presumptive auditory epithelium removed from an embryo of 37½ hours, twelve somites. Fixed after 23¼ hours. A small right ear is present (on the right in figure).

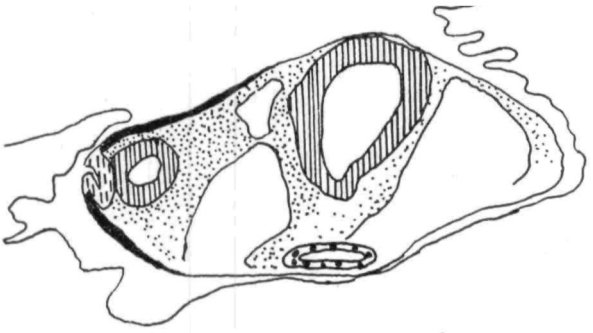

Fig. 3. No. 36–82. The right neural fold was removed from the otic region of a 38-hour, eight-somite embryo, and grafted beneath the ectoderm on the left of the head. It has induced a small otic placode (on left in figure). Cultivation 22 hours.

Before we can conclude, from the eleven embryos which developed ears, that the determination of the ear can be performed by something other than the neural fold, it is necessary to examine the condition of the injured neural fold more carefully. A certain amount of regulation of the defective region would be expected from previous work (Waddington & Cohen, 1936) and certainly occurs. If regulation can replace the missing tissue before it is required to induce the formation of the ear vesicle, the removal of the tissue would not in fact have been effective at the

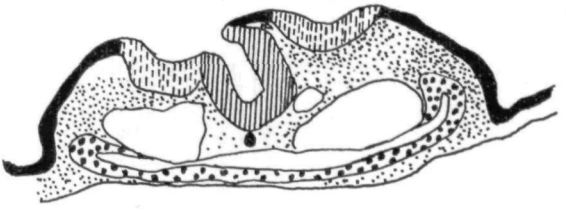

Fig. 4. No. 36–46. The right neural fold was removed from the otic region of a seven-somite embryo. Cultivated 22 hours. A small right otic placode is present (on left in figure). It is joined directly on to the wounded edge of the neural fold in its posterior part, and there is no neural cres in this region and no acoustico-facialis ganglion.

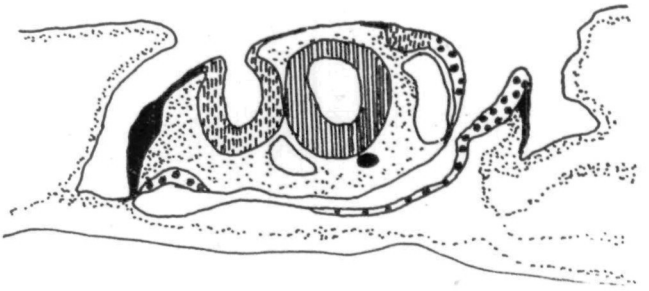

Fig. 5. No. 36–24*a*. The right neural fold was removed, with a little ectoderm, from the otic region of a 50½-hour, eleven-somite embryo, and grafted into the left amnio-cardiac vesicle, where it has formed a large mass of neural tissue with a small otic sac. The rest of the presumptive auditory epithelium was also removed. Only a very minute patch of otic placode is visible in the right ear region after 27 hours' cultivation. It is joined up with the endoderm of the right foregut diverticulum, which has broken through the wound in the ectoderm. The neural tube is nearly normal in cross-section in this region, and it is probable that the acoustico-facialis is present though it is not very clear.

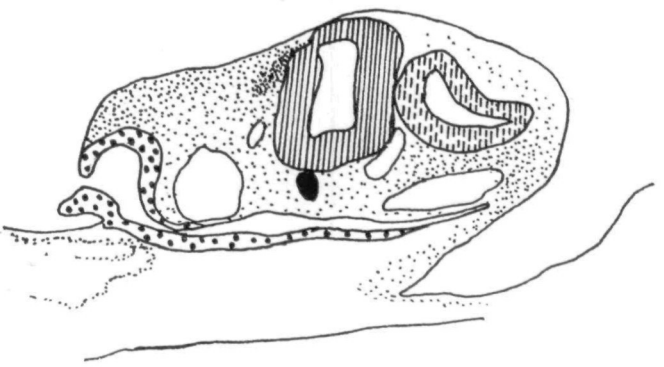

Fig. 6. No. 36–41. The right neural fold was removed from the otic region of a 51½-hour, twelve-somite embryo, and grafted into a hole in the mesenchyme on the left of the head; it has formed a neural tube. The auditory ectoderm was removed to the right amnio-cardiac vesicle and has formed a good otic sac. After 27 hours' cultivation, no ear is present on the right side (left in figure) although the wound in the neural tube is nearly completely healed. The acoustico-facialis is present, apparently derived from the material which is repairing the wound in the neural tube.

crucial stage. The sections show, however, that this was not always the case, even if it ever was. Thus the defects can still be seen in most embryos, the normal cross-section of the neural tube not being completely restored. The question is particularly interesting in relation to the acoustico-facialis ganglion, to which Szepsenwol attributed the induction of the ear vesicle. In some embryos, even in some of those in which the complete cross-section of the neural tube has not been attained, the ganglion is present. In some specimens it appears to be formed from non-presumptive cells from the wound region of the neural plate on that side (Fig. 6), but in others the ganglion cells are derived from the neural crest of the opposite, uninjured side, which have migrated across above the neural tube. In still other embryos the acoustico-facialis is entirely lacking. This is clearest in embryos in which the wounded side of the neural groove has not continued folding up but has united with the ectoderm, leaving the neural groove open (Fig. 4). Such specimens make it impossible to accept Szepsenwol's statement that the acoustico-facialis is the organizer of the ear, if by this he implies that it is the sole organizer.

REMOVAL OF BOTH THE NEURAL FOLD AND THE PRESUMPTIVE EAR ECTODERM

When the presumptive ear ectoderm is removed, the full inducing forces of the embryo are brought to bear on the new ectoderm which grows over the wound; when the neural fold is removed, all the inducing forces minus those of the neural fold act upon ectoderm which, although not capable of developing into an ear vesicle when isolated, must probably be regarded as partly determined to differentiate into an ear since it is already slightly thickened in the first stages of this differentiation. In both cases an ear is formed; in the first case by the full inducing forces acting on indifferent ectoderm, in the second by reduced inducing forces acting on ectoderm which already has a tendency to ear formation. If both the presumptive ectoderm and the neural fold are removed, the residual inducing forces which proceed from parts other than the neural fold act upon ectoderm which has no tendency to become an ear. Ten embryos operated in this way showed scarcely a trace of the operated ear (Figs. 5 and 6), though in some specimens operated on in the seven- and eight-somite stages very minute patches of thickened ectoderm were present in the ear region. One may conclude that the inducing forces which can be exerted by the parts of the embryo other than the neural fold are definitely weaker than those of the intact embryo, and therefore that the inducing capacity of the neural fold itself is not negligible.

INDUCTION OF THE EAR VESICLE BY THE NEURAL FOLD

Since new ear vesicles can be induced in the non-presumptive ectoderm growing over wounds from which the normal ear ectoderm has been removed, it is clear that it should be possible to induce ear formation in a more enlightening way by the grafting of suitable inducing tissue in contact with ectoderm. Since we have seen that the neural fold possesses some capacity for inducing the ear, this tissue

suggests itself as suitable for the grafts. Seventeen grafts have been examined, the grafted tissues being obtained from the defect experiments described in the two previous sections. A few of the grafts were made into extra-embryonic sites, but most were placed in the head, a slit being made in the ectoderm beside the midbrain and the graft inserted into the mesenchyme and covered as well as possible with the cut edges of ectoderm, which soon grow together over the wound.

In some specimens small patches of ear ectoderm have been included with the graft; they develop as small rounded tubular structures. The main part of the graft forms a large mass of neural tissue, usually forming a tube or part of a tube. Only in two cases is an induced ear present, and in both cases it is very small, little more than a slight sac of thickened ectoderm. One of these was in an embryo operated with eight somites, the other in an embryo of six somites. The former (Fig. 3) was a specimen in which the neural fold alone was removed and used as a graft, the presumptive ear ectoderm being left. It provides a clear proof that more than one structure is involved in the induction of the ear, since not only has the grafted neural fold induced an ear, but the structures remaining after the removal of the neural fold have caused the formation of a small ear by the presumptive ear ectoderm, which certainly could not have developed into an ear in isolation.

DISCUSSION

It is clear from the experimental results that several factors must play a part in the determination of the ear vesicle. The wall of the neural tube, including the neural crest, has been shown to be capable of inducing an ear, but so far it has only induced rather small and badly developed vesicles. This probably indicates a real insufficiency of inducing capacity. At any rate it is certain that in normal development the neural tissue is aided in producing an ear by influences proceeding from other neighbouring tissues, presumably from the mesoderm, and these other influences are capable of producing a small ear in the absence of the wall of the neural tube.

The determination which is produced by the co-operation of these tissues seems to arise slowly. This is shown by the gradual increase in the amount of differentiation obtained in isolated presumptive ear ectoderm as it is taken from progressively older embryos. One must also assume that the determination is beginning even in stages younger than the youngest from which self-differentiation of the isolates can be obtained. This is shown by the fact that in embryos of five or six somites, if the wall of the neural tube is removed, the remaining tissues can produce an ear from the presumptive ear ectoderm though not from non-presumptive ectoderm, although the isolated ectoderm will not develop into an ear; even at this stage then the presumptive ectoderm forms an ear more easily than does the non-presumptive ectoderm.

The present results do not justify one in denying that the acoustico-facialis ganglion may play a part in the determination of the ear, but they do demonstrate that it is not the only factor concerned. This is conclusively shown by the occurrence of ears in embryos in which the ganglion is entirely absent on the operated side.

The general picture of the determination of the ear of the chick, as a process involving the simultaneous action of many different factors, is similar to that which has been suggested for the amphibian ear by Holtfreter (1933) and Dalcq (1933). Holtfreter in particular has shown that not only are there several tissues which co-operate to produce the normal ear but that each tissue separately is quite able to induce an ear without the help of the others. The ear, in fact, seems to be one of the most easily induced structures in amphibian embryos. Although one cannot give any quantitative estimate, it seems that in the chick the ear is much less easily produced, and that a fully developed ear of normal size is only produced when all the co-operating factors combine to induce it.

SUMMARY

1. The presumptive ear ectoderm, removed from its normal site and transplanted to the amnio-cardiac vesicle, does not develop into an otic placode unless it comes from an embryo with more than nine pairs of somites.

2. The ectoderm which grows over the place from which the presumptive ear ectoderm is removed is induced to form an otic placode, the size of the placode being smaller the older the embryo at the time of operation.

3. If the wall of the neural tube, including the neural crest, is removed from the region of the ear in embryos younger than the nine-somite stage, an ear may nevertheless be formed. Since the ear ectoderm at this stage is not capable of differentiating when isolated, this result shows that inducing agencies other than the neural material are active at this stage. In some of the operated embryos, the acoustico-facialis ganglion was completely lacking, so this structure cannot be the sole organizer of the ear, as Szepsenwol suggested.

4. If both the wall of the neural tube and the presumptive ear ectoderm are removed, no ear is formed even in stages younger than the nine-somite stage, so that it appears that the non-neural inducing agents are more effective when working on presumptive ectoderm of this age than when working on non-presumptive ectoderm. This suggests that the ear ectoderm is beginning to be determined some time before it acquires the capacity for independent differentiation.

5. The wall of the neural tube, from the ear region, can induce a small ear when grafted under the ectoderm of the anterior part of the head.

6. The evidence suggests that the induction of the ear in normal development is due to the combined action of several structures, of which the wall of the neural tube, and the tissues derived from it (ganglia), is one but not the only one.

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