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Retinoic Acid Disturbs Mouse Middle Ear Development in a Stage-Dependent Fashion

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The mammalian middle ear contains a chain of three ossicles, the malleus, incus, and stapes, that transmit into the inner ear the vibrations produced in the tympanic membrane by aerial sound. I show here that retinoic acid interferes with the formation of the middle ear in a stage-specific fashion. The malformations produced are derived from a retinoic acid-induced inhibition of the formation and/or migration of the cranial neural crest that generates the middle ear skeletal elements and not from a respecification of neural crest identity to more posterior fates. I have used these effects of retinoic acid to analyze the temporal sequence of events involved in the morphogenesis of the middle ear. I show that the middle ear bones develop from several primordia that originate in a typical temporal sequence from Day 8 plus 4.5 hr to Day 8 plus 7.5 hr of pregnancy. Moreover, interactions between adjacent bones are not required for their proper formation. My results also suggest a *Hoxa-2*-mediated patterning of the otic capsule in the region where the oval window is located.

INTRODUCTION

The cranial neural crest cells emerge from the juncture of the ectoderm and neuroectoderm in the rostral neural folds and migrate lateroventrally into the branchial arches and the frontonasal mass of the developing vertebrate embryo (Lumsden *et al.*, 1991; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). These cells undergo a complex series of interactions to give rise to a wide variety of derivatives including neurons, glia, dermis, bone, cartilage, and connective tissue of the face (Le Dourain, 1982; Noden, 1983). The contribution of the neural crest to craniofacial morphogenesis is very extensive. For instance, it has been established, mainly by the use of chick-quail chimeras, that the facial skeleton of higher vertebrates is entirely derived from the neural crest (Le Dourain *et al.*, 1993).

The neural crest cells are regionalized in both their migratory patterns and their differentiation potential. Crest cells arising from different rostrocaudal levels in the neural tube migrate into very specific regions of the developing face (Lumsden *et al.*, 1991; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). From each of these structures characteristic sets of derivatives develop. In particular, the skeletal components of the middle ear develop from the first and second branchial arch neural crest (Moore and Persaud, 1993). The tympanic ring, which supports the tympanic membrane, and the malleus and incus, which are involved in the transmission of aerial sound through the middle ear, are all first arch derivatives; the stapes, the third of the sound-transmitting ossicles, derives from the second branchial arch. The molecular and cellular mechanisms responsible for this regional specification are still not clear. It is generally accepted, at least as far as the skeletogenic neural crest is concerned, that epithelial–mesenchymal interactions are of crucial importance for the development of the skeletal elements from the neural crest cells (Le Dourain, 1982). It is not clear, however, whether these interactions act by unmasking a developmental prepattern that was given to the cells before their migration or if they pattern themselves.

The first alternative is supported by grafting experiments in the avian system and by gene inactivation experiments in mouse. First, when premigratory neural crest was transplanted heterotopically, it migrated as expected for crest cells arising from the host location but then developed skeletal structures characteristic of the region into which the grafted crest would migrate in their donor embryos (Noden, 1988); second, the inactivation of *Hoxa-2*, which is normally expressed in the migratory second arch neural crest, resulted in an anterior transformation in which a duplicated set of typical first branchial arch skeletal elements developed from the second arch region (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). These results indicated that *Hoxa-2* acts as a selector gene that patterns the second arch skeletogenic neural crest. The other hypothesis gains some support from the analysis of the expression of extracellular

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matrix components in the developing face and upon mesenchymal–epithelial recombinations and from the effects of that extracellular matrix on the mobility of the neural crest *in vitro* (Thorogood, 1988). In these experiments it was shown that type II collagen is expressed in a spatial and temporal pattern that correlates with the sites of chondrogenesis in the developing face (Thorogood, 1988). Moreover, matrices derived from those chondrogenic sites were able to cause arrest of neural crest mobility *in vitro*. From those results it was suggested that freely migrating neural crest becomes entrapped in and is induced to differentiate by the matrix synthesized at epithelial–mesenchymal interfaces at the prospective sites of chondrogenesis.

These two hypotheses are not necessarily mutually exclusive and it is possible that a combination of both mechanisms exists, each being involved in the formation of particular structures or of specific regions along the rostrocaudal axis. Another question is whether the cranial neural crest cells are prespecified to particular fates (e.g., cartilage or neurons) before they migrate or, as usually happens with the trunk neural crest cells (Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991; Le Dourain and Dupin, 1992; Riable and Eisen, 1994), they acquire their final identity in the target location. Although no final answer has been reached yet, the results obtained in chicken (Le Dourain *et al.*, 1993; Gale *et al.*, 1996) and zebrafish (Schilling and Kimmel, 1994) seem to favor the first hypothesis.

Recent work using *in vitro* cultured explants and gene inactivation by homologous recombination has led to the identification of an increasing number of genes involved in various steps of neural crest differentiation (Chisaka *et al.*, 1992; Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993; Vainio *et al.*, 1993; Lohnes *et al.*, 1994; Satokata and Maas, 1994; Kurihara *et al.*, 1994; Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Martin *et al.*, 1995; Matzuk *et al.*, 1995; Qiu *et al.*, 1995). Some of these have been shown to have a role in the morphogenesis of the middle ear skeletal elements. For instance, mutations in the above-mentioned *Hoxa-2* (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993), *Hoxa-1* (Chisaka *et al.*, 1992), *goosecoid* (*gsc*) (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995), *endothelin-1* (*ET-1*) (Kurihara *et al.*, 1994), *Mhox* (Martin *et al.*, 1995), *Msx-1* (Satokata and Maas, 1994), or *Dlx-2* (Qiu *et al.*, 1995) all result in different phenotypic changes in the ear region. Moreover, the correct spatiotemporal expression of some of these genes directs particular morphogenetic events that eventually lead to the formation of a functional tympanic membrane (Mallo and Gridley, 1996).

Retinoic acid (RA) has attracted the attention of embryologists during the past decades because of its importance in normal embryonic development (Wilson *et al.*, 1953; Lohnes *et al.*, 1994) and its teratogenic effects when administered during particular times of development (Kochhar, 1967). The teratogenicity of RA has been used extensively to study several aspects of vertebrate development (Sive and Cheng, 1991; Kessel and Gruss, 1991; Kessel, 1992, 1993; Marshall *et al.*, 1992; Niederreither *et al.*, 1996). In this paper I show that, when administered to pregnant females at particular time points during pregnancy, RA induces stage-specific alterations in the middle ear of the mouse as a consequence of a drug-mediated inhibition of formation and/or migration of the neural crest. I analyze the phenotypes resulting from RA treatments at slightly different time points of development and discuss their relevance for understanding ear development.

MATERIALS AND METHODS

(C57BL/6 × DBA/2)-F1 mice were mated for 2 hr. When plugs were detected, the half-time of the mating period was considered as the time of fertilization. All-*trans*-retinoic acid (Sigma) was administered dissolved in sesame oil at a concentration of 20 mg/kg by oral gavage. I will refer to the time of the administration of the drug as days + hr. The *Hoxa-2* mutant embryos were genotyped by PCR as described (Gendron-Maguire *et al.*, 1993).

Whole-mount *in situ* hybridization was performed as described in Wilkinson (1992). The probe for *CrabpI* consisted of a 750-bp cDNA clone containing the entire coding sequence (Stoner and Gudas, 1989). The probe for *Hoxa-2* consisted of a 1.3-kb cDNA fragment containing the whole coding sequence (Tan *et al.*, 1992).

For histological analysis, embryos were obtained at 17.5 days postcoitum (dpc) by cesarean section, fixed in Carnoy's fixative, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin.

All the anatomical orientations throughout this paper will be described according to the following nomenclature: superior and inferior will refer to the axis from the vault of the skull to the neck; medial and lateral to the axis defined by the two ears, medial being the center of the head and lateral both ear auricles; and rostral and caudal to the axis from the snout to the occipital region of the skull.

RESULTS

Retinoic Acid Effects on First Branchial Arch Neural Crest Identity

It has been previously shown that the treatment of pregnant females with RA at Day 8 + 5 hr of gestation leads to a failure of the tympanic ring to develop (Kessel, 1992; Mallo and Gridley, 1996). A preliminary histological analysis of the ear region of 17.5 dpc embryos subjected to this treatment revealed additional anomalies in the skeletal elements of the middle ear: the typical second branchial arch derivative in this region, the stapes, appeared to be present as a cartilaginous formation in the oval window; however, the typical ear skeletal elements derived from the first branchial arch, namely malleus, incus and tympanic ring, were not readily identifiable, although some cartilaginous formations were seen in this region (Mallo and Gridley, 1996; data not shown, and see later). It has been extensively reported that RA treatments are able to induce transformations in anterior–posterior segment identity of the neural tube and mesenchymal axial elements. Normally RA causes anterior seg-

ments to adopt posterior features. The proposed genetic mechanism for such a transformation was a RA-induced anteriorization of *Hox* gene expression (Kessel and Gruss, 1991; Kessel, 1992, 1993; Marshall *et al.*, 1992; Hill *et al.*, 1995). *Hoxa-2* has been shown to be responsible for the specification of the second arch skeletal derivatives (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Therefore, I asked whether RA also affected the middle ear by modifying the expression of *Hoxa-2* to respecify the first arch neural crest to a second arch identity. To check this possibility, I first analyzed the effects of this RA treatment on *Hoxa-2* expression. In the branchial area, this gene is normally expressed in the second but not the first branchial arch (Krumlauf, 1993; Prince and Lumsden, 1994; and Fig. 1A), a pattern that reflects its functional domain. If a posterior transformation occurs in this area, it would be reflected in an anteriorization of *Hoxa-2*. However, of the 24 embryos treated with RA at this stage that I have analyzed, none showed induction of *Hoxa-2* in the first branchial arch (Fig. 1B). This argues against the respecification hypothesis. As a further test of this hypothesis, I analyzed the ear region of the progeny from *Hoxa-2*$^{+/-}$ intercrosses after treatment of the pregnant females with RA under the same conditions as above. In the case of a respecification, I would expect that homozygous mutants that were exposed to RA would show no differences with respect to the control untreated homozygous embryos. However, in all four cases, RA treatment resulted in hypomorphic phenotypes in this area (Figs. 1C–1F). There was a variable loss of ear skeletal structures and both sets of typical first arch derivatives that develop from the first and second branchial arches of the *Hoxa-2* mutants (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993) were affected (see Figs. 1C–1F for details on an example). Thus, these experiments indicate that a respecification of the anterior–posterior identity in the branchial arch region is not likely to be responsible for the ear defects arising from the RA treatment.

RA Effects on Neural Crest Formation/Migration

An alternative explanation is that RA induced the ear phenotype through an inhibition of the formation and/or migration of the neural crest that generates the middle ear bones. In fact, RA has been shown to affect the mobility of the neural crest (Thorogood *et al.*, 1982), and the RA treatment was performed when the neural crest cells that populate the region of the branchial arches were migrating (Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). To test this possibility, I checked for the presence of migratory neural crest from the hindbrain 2 hr after the RA treatment, using *CrabpI* as a marker. In accordance with published data, at this developmental stage this probe labeled a stream of neural crest cells arising from the hindbrain (Maden *et al.*, 1992; and Fig. 2A). In the RA-treated embryos, however, this stream was not evident (Fig. 2B). This is consistent with the hypothesis that the RA treatment inhibits the formation and/or migration of the cranial neural crest. This inhibition is reversible since neural crest cells could be detected again 24 hr after the RA treatment (Figs. 2C and 2D).

Stage-Dependent Effects of RA on Middle Ear Development

Altogether, the above data strongly suggest that the ear phenotype derived from the RA treatment at this gestation time is a consequence of stage-specific inhibition of the neural crest that gives rise to these skeletal structures rather than of a posterior transformation of the first branchial arch. A possible consequence of this physiopathological mechanism is that variations in the time of exposure to the drug would result in different phenotypic effects. This would also explain the variable phenotypes I have observed in some preliminary experiments. Therefore, I treated pregnant females with RA at half-hour intervals from Day 8 + 4 hr to Day 8 + 8 hr. The analysis of the ear region of these embryos showed phenotypes that ranged from a complete lack of middle ear skeletal elements to fairly normal middle ears. These phenotypes can be ordered in a temporal sequence in which more elements are added sequentially to the previous pattern. I will describe first the typical ear pattern of a normal 17.5 dpc embryo and then the seven different patterns I have seen on the RA-treated embryos, proceeding from less to more complex (summarized in Fig. 5).

Normal pattern. Going from lateral to medial, we first find the tympanic membrane, which is supported on the tympanic ring (Figs. 3A and 3B). The first middle ear ossicle, the malleus, is attached to the tympanic membrane through its manubrium (Fig. 3A). The neck of the malleus connects the manubrium to the body of the ossicle (Fig. 3B), still attached to the caudal extremity of Meckel's cartilage at this stage of development (not shown). A joint is then established between the bodies of the malleus and of the next ossicle, the incus (Fig. 3B), whose long process is connected to the third of the ossicles, the stapes (Figs. 3C and 3D). The stapes consists of two elements, the footplate, which is located in the oval window, and the arch, articulated to the incus, that delimits the stapedial foramen which is crossed by the stapedial artery (Fig. 3D).

Pattern 1. In this case there was a complete lack of all the ear skeletal elements. There was an opening in the otic capsule at the position where the oval window is normally located (Fig. 4A; compare with Fig. 3D). It was smaller than the typical oval windows found in untreated control animals and no stapedial footplate could be found in it. The caudal extremity of Meckel's cartilage usually ended with a smooth thinning (not shown), although sometimes a lateral thickening on the caudal extremity of Meckel's cartilage was present (Fig. 4B). This could be regarded as a small primordium of the body of the malleus.

Pattern 2. In the caudal extremity of Meckel's cartilage, a more complex cartilaginous formation was present (Fig. 4C). There was a bigger enlargement in the caudal extremity of Meckel's cartilage, which was articulated to another more caudally located cartilage, whose caudal extremity

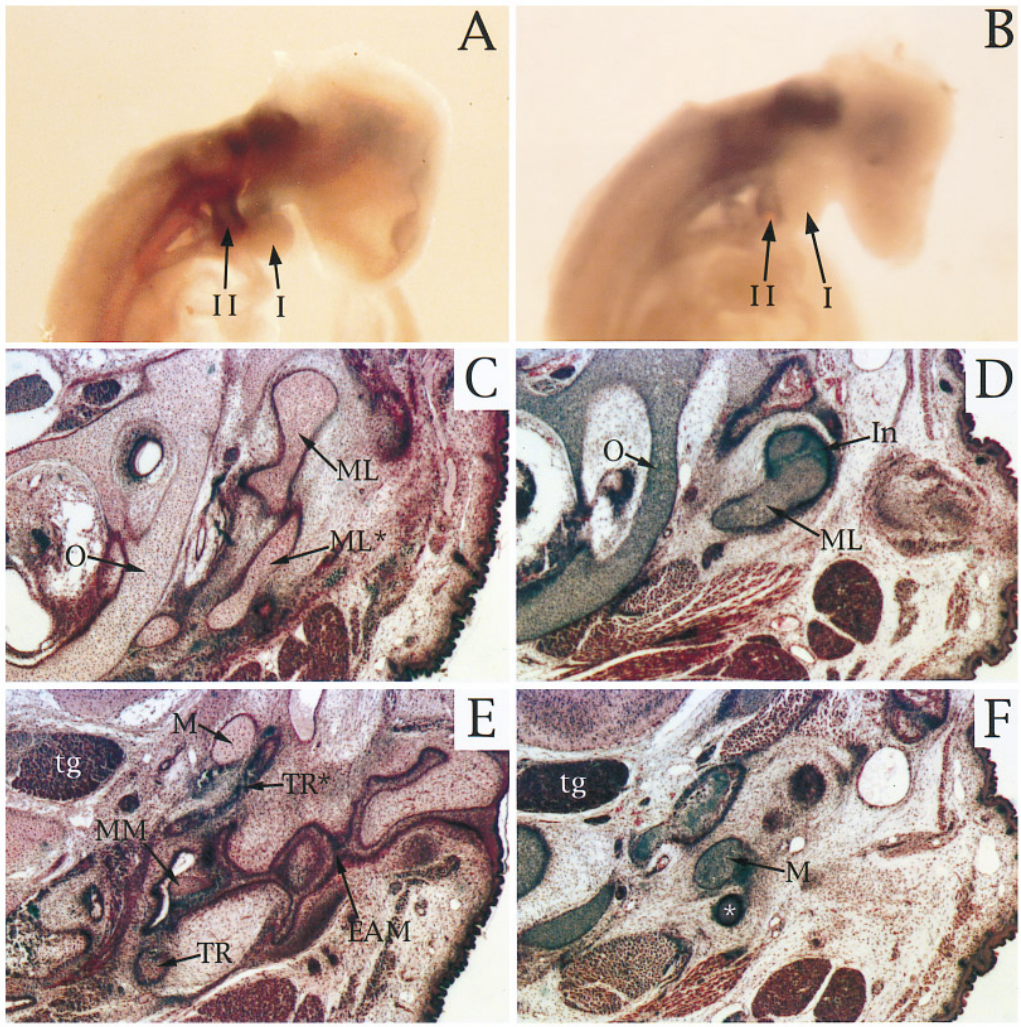

FIG. 1. Effects of RA on *Hoxa-2* mRNA expression in wild-type and *Hoxa-2* null mutant embryos. (A, B) Wild-type embryos from control untreated (A) or RA-treated mothers at Day 8 + 5 hr of pregnancy (B) were analyzed for mRNA by whole-mount *in situ* hybridization at 9.5 dpc with an antisense probe for *Hoxa-2*. In both cases, the rostral limit of the expression of this gene in the arch region is the second branchial arch (II), the first arch (I) being completely devoid of hybridization to this probe. (C–F) Histological analysis of the ear region of 17.5 dpc *Hoxa-2*$^{-/-}$ embryos from control untreated (C and E) or RA-treated mothers at Day 8 + 5 hr of pregnancy (D and F). Frontal sections through two matching areas (C with D and E with F; C and D being more caudally located) are shown for comparison. At the dorsal level, in the control *Hoxa-2* mutant embryo (C) two malleus are seen lateral to the otic capsule (O), one derived from the first branchial arch (ML) and the other from the transformed second branchial arch (ML*); in the RA-treated embryo (D) the duplicated malleus is absent and only the one derived from the first arch is present. A small portion of the incus (In) is also seen articulated to the malleus. At the rostral level, the control *Hoxa-2* mutant (E) shows a duplicated tympanic ring (TR and TR*, derived from the first and second branchial arches) and an external acoustic meatus (EAM) with a duplicated medial end duplicated directed toward each of the tympanic rings to generate an abnormal tympanic membrane that encloses the manubrium of the malleus (MM). In the RA-treated embryo, none of these structures was present. A small cartilage (white asterisk), which I could not identify, was seen close to Meckel's cartilage (M). All the sections are oriented with the lateral side to the right and the superior to the top. tg, trigeminal ganglion.

finished free in the surrounding soft tissue (not shown). From their anatomical relationships, these two new cartilaginous formations can be regarded as primordia for the bodies of the malleus and incus. The rest of the otic region looked similar to that in pattern 1.

Pattern 3. This pattern differed from the previous one in that the oval window was now occupied by a cartilage that clearly resembled the stapedial footplate (Fig. 4E; compare with Fig. 3D). However, no stapedial arch was present. In one case, instead of a clear footplate, a small cartilage

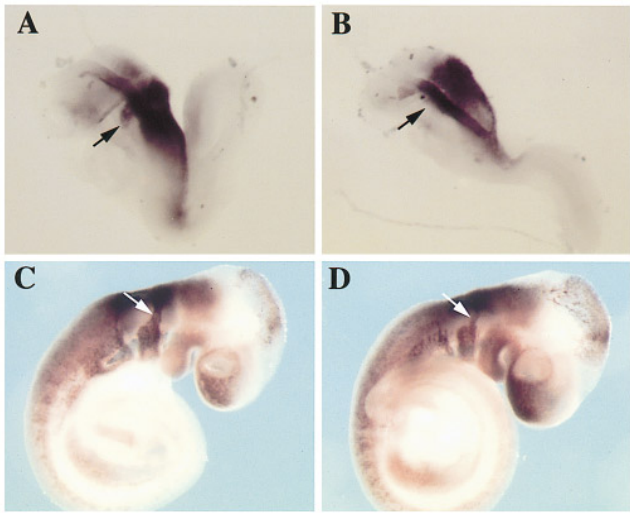

FIG. 2. Effects of RA on the expression of *CrabpI*. Embryos from wild-type pregnant females, untreated (A and C) or treated with RA at Day 8 + 5 hr of pregnancy (B and D), were analyzed for their expression of *CrabpI* by whole-mount *in situ* hybridization at Day 8 + 7 hr (A and B) or Day 9 + 5 hr (C and D) of development. The black arrows in A and B indicate the stream of neural crest cells emerging from the hindbrain (missing in B). The white arrows in C and D indicate a stream of neural crest cells migrating into the second branchial arch.

was seen in close association with the oval window (Fig. 4D). I consider it a small primordium of the stapedial footplate.

Pattern 4. This pattern was somewhat more complex with several new elements present. In the region of the oval window it was similar to pattern 3 (not shown). The skeletal formations on the caudal extremity of Meckel's cartilage described in pattern 2 were still present, although they were clearly bigger now (Fig. 4F and not shown). Three new elements were found in these embryos (Fig. 4F; compare with Fig. 3B): (i) an ossified element that, by its shape, general position, and type of ossification (endomembranous), can be identified as a tympanic ring; however, it was smaller and located closer to the Meckel's cartilage than the typical rings from the control embryos (not shown); (ii) an external acoustic meatus which, as has been previously described (Mallo and Gridley, 1996), developed in close morphogenetic association with the tympanic ring; and (iii) an elongated cartilage, devoid of any contact with other skeletal formations. This last element was located along the rostrocaudal axis of the head with a slight inferior–superior inclination, situated on the medial surface of the external acoustic meatus, close to the concave edge of the tympanic ring. Considering its anatomical position, I suggest that this cartilage represents the manubrium of the malleus.

Pattern 5. This pattern was similar to pattern 4, but a complete stapes, with a full arch attached to the footplate enclosing the stapedial artery, was present in the oval window (Fig. 4H; compare with Figs. 3C and 3D). In one case, instead of a normal-looking stapedial arch, a small, rod-shaped cartilage was present just lateral to the stapedial artery, in close proximity to the stapedial footplate with which it did not establish any contact (Fig. 4G). From its position, it might be a primordium for the stapedial arch.

Pattern 6. A nearly complete ear was seen (Figs. 4I and 4J). The manubrium and body of the malleus were connected by a neck (Fig. 4I; compare with Fig. 3B). The malleus, still attached to the caudal extremity of Meckel's cartilage, was articulated to an incus that was still not touching the normal-looking stapes. Interestingly, the external acoustic meatus seemed to be ''flattened'' on the imaginary surface enclosed by the tympanic ring, also in close juxtaposition with the manubrium of the malleus. This generated a structure resembling the lateral half of an eardrum (Fig. 4J; compare with Fig. 3A). However, there was no clearly organized tympanic membrane, very likely because the tubotympanic recess, which contributes to the eardrum with its lateral surface, had not formed at all or was very reduced (Fig. 4J and data not shown).

Pattern 7. This was a complete, normal-looking middle ear with all its components (not shown).

In general, no single phenotypic pattern could be ascribed to any particular time of treatment (Table 1). Typical patterns were only seen after the earliest (Day 8 + 4 hr and Day 8 + 4.5 hr) and the latest (Day 8 + 7.5 hr and Day 8 + 8 hr) treatments in which I have always found patterns 1 and 7, respectively. However, there was a clear trend that the later the RA treatment, the more ear skeletal elements were present. It is also noteworthy that often the patterns found in embryos from the same litter were different, which might reflect the small differences in developmental stages within a given litter. In many cases, even both sides of the same embryo were different, although always within a ''two-pattern difference.''

DISCUSSION

The teratogenic effects of RA, which have been extensively reported in a wide variety of vertebrates (Kochhar, 1967; Shenefelt, 1972; Fantel *et al.*, 1977, Lammer *et al.*, 1985; Webster *et al.*, 1986), have been used as a tool to study different aspects of embryonic development (Sive and Cheng, 1991; Kessel and Gruss, 1991; Kessel, 1992, 1993; Marshall *et al.*, 1992, Niederreither *et al.*, 1996). In this study, I describe steps in the morphogenesis of the middle ear bones taking advantage of time-dependent alterations of these bones resulting from the administration of RA to pregnant mice during the first hours of the ninth day of pregnancy. In previous studies, it has been shown that this particular treatment induces a number of other malformations. Webster *et al.* (1986) have shown that it induces several craniofacial abnormalities, among which the most salient are different degrees of malformations of the auricle

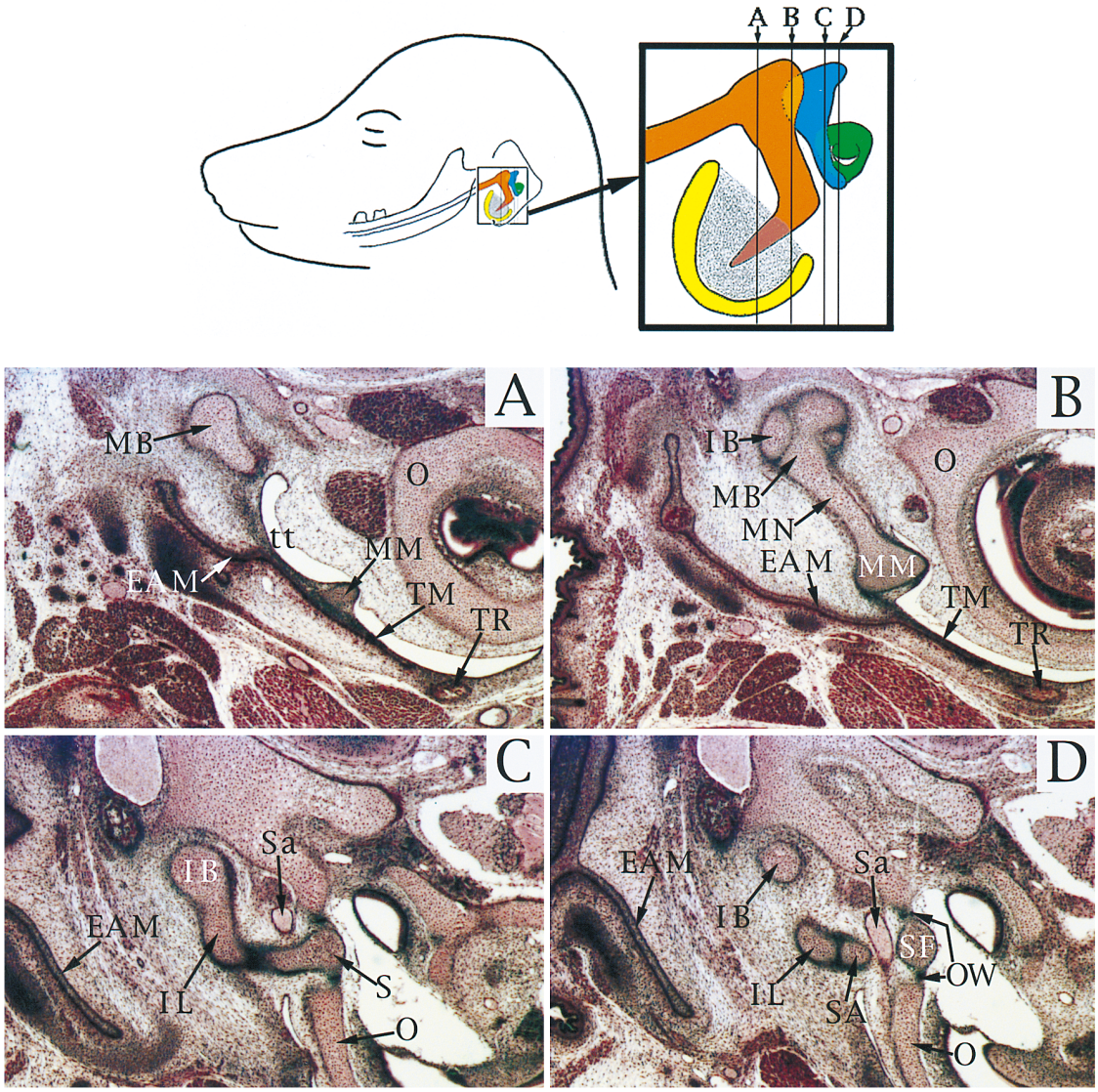

FIG. 3. Middle ear region from a normal 17.5 dpc mouse embryo. Sections through four frontal planes (A to D from rostral to caudal; see diagram for orientation) of the otic region of a 17.5 dpc mouse embryo are shown, stained with hematoxylin and eosin. They illustrate the typical elements of a normal middle ear: a tympanic membrane (TM), formed by the aposition of the epithelia from the external acoustic meatus (EAM) and the tubotympanic recess (tt), is supported on the tympanic ring (TR). The manubrium of the malleus (MM) is inserted in this membrane and is connected to the body of the malleus (MB) by a neck (MN). The body of the malleus is articulated with the body of the incus (IB) which connects with the stapes (S) by its long process (IL). The stapes consists of an arch (SA), articulated to the incus, and a footplate (SF) which is located in the oval window (OW) of the otic capsule (O). The stapedial footplate and arch define a foramen that is crossed by the stapedial artery (Sa). All the figures are oriented with the lateral side to the left and the superior to the top. In the diagram, the malleus is in orange, the incus in blue, the stapes in green, the tympanic ring in yellow, and the tympanic membrane in gray.

of the ear, and anomalies in internal organs like the thymus and the aortic arch. This picture closely resembles the RA embryopathy described after the accidental exposure of human embryos to this drug (Lammer *et al.*, 1985). Later, Kessel (1992) reported that this RA treatment also induces posterior transformations of the axial skeleton in the upper cervical region and the base of the cranium. The mechanisms through which RA was proposed to induce both kinds of malformations were, however, different in the two studies. Kessel (1992) explained the phenotype as a change in segment identity derived from a RA-induced anteriorization of *Hox* gene expression. Webster *et al.* (1986) suggested,

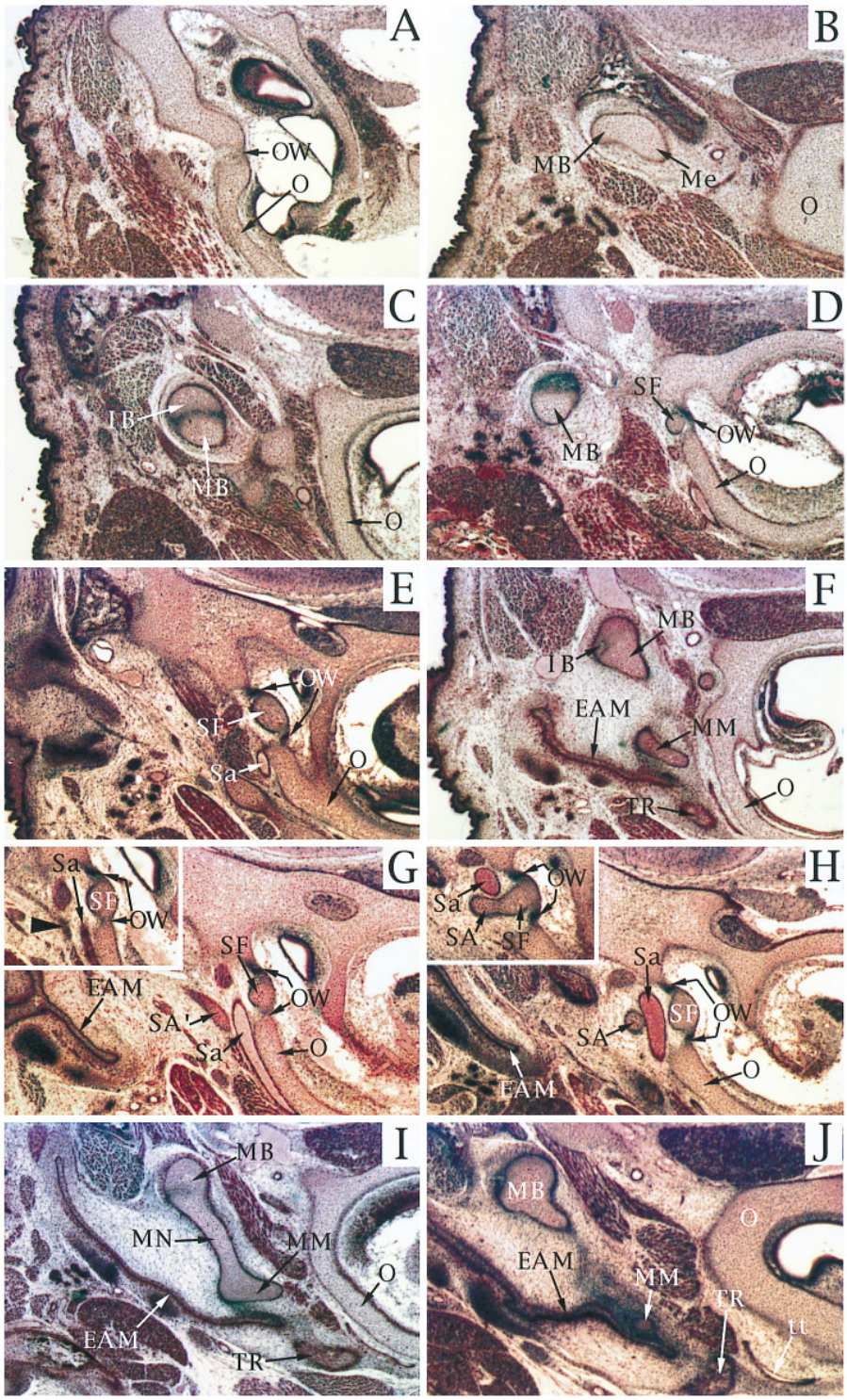

FIG. 4. Phenotypic changes in the middle ear derived from the RA treatments. Frontal sections through the ear region of 17.5 dpc embryos from females that were treated with RA at different time points from Day 8 + 4 hr to Day 8 + 8 hr of pregnancy are shown. Each of the pictures illustrates features that were new for each phenotypic pattern. (A) An empty oval window (OW) is seen in the otic capsule (O) (from pattern 1). (B) A primordium of the body of the malleus (MB) is seen laterally attached to the caudal extremity of Meckel's cartilage (Me) (pattern 1). (C) The primordium of the body of the malleus (MB) is articulated to a primordium of a body of the

TABLE 1
Correlation between Treatment Times and Ear Patterns

	Time of retinoic acid treatment (days + hours)								
	8 + 4	8 + 4.5	8 + 5	8 + 5.5	8 + 6	8 + 6.5	8 + 7	8 + 7.5	8 + 8
Pattern 1	3	4	2	0	0	0	0	0	0
Pattern 2	0	0	2	2	0	0	0	0	0
Pattern 3	0	0	3	3	1	0	0	0	0
Pattern 4	0	0	1	2	3	2	0	0	0
Pattern 5	0	0	0	0	1	3	0	0	0
Pattern 6	0	0	0	0	1	3	3	0	0
Pattern 7	0	0	0	0	0	0	3	6	4

Note. Expressed in number of embryos in which a particular pattern was found. When an embryo presented different patterns in each side it was scored in both categories.

from morphological studies using scanning electron microscopy, that the craniofacial abnormalities were a consequence of a RA-induced impairment of the migratory ability of the cranial neural crest, a conclusion also supported by studies on *in vitro* cultured mouse embryos (Pratt *et al.*, 1987).

RA Affects the Formation/Migration of the Neural Crest but Not Its Identity

In the case of the middle ear, the phenotype also arises from the lack of migration and/or formation of the neural crest that generates the middle ear bones. Using a molecular marker, I have found a lack of cranial neural crest in the RA-treated embryos within the time period of the action of the drug (Creech-Kraft *et al.*, 1987) and during the time when the crest cells migrate to populate the branchial arches (Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). This finding is consistent with the electron microscopy data (Webster *et al.*, 1986; Pratt *et al.*, 1987) and with experiments showing that RA inhibits the migratory ability of the neural crest cells in culture (Thorogood *et al.*, 1982). However, it contrasts with recent reports showing that RA modifies the patterns of neural crest migration more than its migratory ability (Lee *et al.*, 1995; Gale *et al.*, 1996). This discrepancy can be explained considering the time points at which the behavior of the neural crest cells was analyzed in the different studies. I have found inhibition of neural crest formation and/or migration 2 hr after the administration of the drug to the mothers and thus within the active phase of RA (Creech-Kraft *et al.*, 1987). In the other studies, the migration of the neural crest was analyzed 24 hr after an acute exposure of the embryos to the drug (Lee *et al.*, 1995; Gale *et al.*, 1996). As the effects of RA on the mobility of the neural crest are reversible (Fig. 2; Thorogood *et al.*, 1982), what they might have described are the effects of the drug on the migration of the neural crest cells that were generated after the acute phase of exposure to RA. I cannot be certain about the fate of these late migrating crest cells. However, considering that the altered neural crest migration correlated with abnormal morphologies of cranial ganglia (Lee *et al.*, 1995; Gale *et al.*, 1996) and normal mesenchymal derivatives (Gale *et al.*, 1996), and that the mesenchymal and neurogenic crest cells seem to migrate respectively around early Day 8 and 9 of development (Theiler, 1972; Morriss-Kay and Tan, 1987), I suggest that they contributed to the formation of neural structures.

The analysis of migratory neural crest cannot definitively

incus (IB) (pattern 2). (D) A small primordium of the stapedial footplate (SF) is seen in the oval window (OW) (pattern 3). (E) A well-formed stapedial footplate (SF) is located in the oval window (OW); Sa indicates the stapedial artery (pattern 3). (F) Lateral to the otic capsule (O) a body of the malleus (MB) is articulated to the body of the incus (IB); also evident are an external acoustic meatus (EAM) whose medial extremity lies close to the primordium of the manubrium of the malleus (MM) and a tympanic ring (TR) (pattern 4). (G) A primordium of the stapedial arch (SA′) is located lateral to the stapedial artery (Sa); the inset, from a section located 50 μm caudally, shows that this primordium ends free in the surrounding soft tissue (arrowhead) without contact with the stapedial footplate (SF) (pattern 5). (H) A complete stapedial arch (SA) is seen which, along with the stapedial footplate (SF), delimits a foramen crossed by the stapedial artery (Sa); the inset, in a section located 50 μm rostrally, shows the contact between the stapedial arch and footplate (pattern 5). (I) The manubrium and body of the malleus (MM and MB, respectively) are connected by a neck (MN); the external acoustic meatus is seen in close proximity to the manubrium of the malleus and the tympanic ring (TR) (pattern 6). (J) The external acoustic meatus (EAM) flattens medially in close anatomical position to the manubrium of the malleus (MM); the medial edge of the meatus is supported on the tympanic ring; the tubotympanic recess (tt) is very small and located far from the external acoustic meatus. All the figures are oriented with the lateral side to the left and the superior to the top.

distinguish whether all the neural crest is affected or only a portion and, therefore, is not sufficient to rule out a contribution of the late migrating neural crest to the middle ear structures. The ear phenotype could thus be a consequence of a posterior transformation of the skeletogenic neural crest that migrates into the branchial arches after the recovery of the neural crest from the effects of RA. However, from the analysis of *Hoxa-2* expression in RA-treated embryos and the study of the effects of the drug on the *Hoxa-2* mutants, I conclude that RA could not have induced a posterior transformation in the arch region. Genetic data have clearly established that *Hoxa-2* is the key gene in the specification of the second branchial arch skeletogenic neural crest (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Therefore, RA should cause such a posterior transformation through an anterior induction of *Hoxa-2*. However, contrary to what has been reported for a number of *Hox* genes (Kessel and Gruss, 1991; Conlon and Rossant, 1992; Kessel, 1992, 1993; Marshall *et al.*, 1992; Wood *et al.*, 1994), the expression of *Hoxa-2* seems not to be affected by this RA treatment. This argues against a posterior transformation in the branchial arch region. Furthermore, I have seen that RA induces deviations of the basic ear phenotype in *Hoxa-2*$^{-/-}$ embryos with a pattern that could be explained by a deficiency in neural crest migration, while no differences between the RA-treated and untreated *Hoxa-2* mutants would be expected in the case of a posterior transformation.

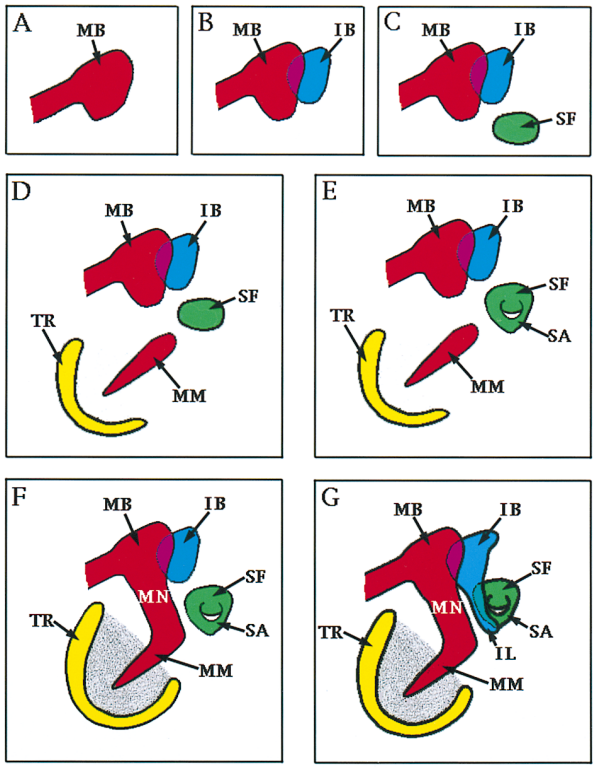

FIG. 5. Schematic representation of the patterns. (A) Pattern 1; (B) pattern 2; (C) pattern 3; (D) pattern 4; (E) pattern 5; (F) pattern 6; (G) pattern 7. IB, body of the incus; IL, incudial long process; MB, body of the malleus; MM, manubrium of the malleus; MN, neck of the malleus; SA, stapedial arch; SF, stapedial footplate; TR, tympanic ring.

Middle Ear Morphogenesis

The middle ear is affected by RA in a stage-specific way. RA treatment from Day 8 + 4 hr to Day 8 + 8 hr of pregnancy has resulted in ear phenotypes ranging from a complete absence of middle ear skeletal elements to fairly normal middle ears. The phenotypes can be ordered in a temporal sequence in which more elements are sequentially added to the previous pattern (Fig. 5). The increase in middle ear elements correlates with later RA administrations (Table 1). This could indicate that RA has an effect on the neural crest cells before or while they migrate from the hindbrain, but that it has no effect on their differentiation after they have reached the branchial arches. Thus, the order of appearance of the middle ear elements would represent the time of migration of the neural crest that generate them. This might reflect a timely predetermination of the neural crest cells that form particular skeletal elements. Alternatively, neural crest cells that had not been predetermined might undergo a time-controlled differentiation in the branchial arch region. Although I cannot distinguish between these two possibilities, grafting experiments performed in avian systems have suggested a prepatterning of cranial neural crest cells at this axial level (Noden, 1988); this would favor the first explanation. The differences found between the RA-treated and the *Hoxa-2* mutant embryos in the region of the oval window (see later) also support the prepatterning hypothesis.

Another interesting aspect of the experiments reported in this paper is that frequently both sides of the same embryo have reached different stages of ear development. This suggests that the developmental program of these crest cells is set independently in each side.

In the most severe phenotype, none of the skeletal elements that form the middle ear region was present. Interestingly, in the absence of stapes, a structure that according to its anatomical position could be considered an oval window was present in the otic capsule. This finding contrasts with the lack of an oval window in the *Hoxa-2*$^{-/-}$ embryos in which the stapes also fails to develop (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). This would imply that the oval window [the region of the otic capsule where it is located might be of neural crest origin (Couly *et al.*, 1993)] is also patterned and does not differentiate only as a consequence of interactions with the Reichert's cartilage. Furthermore, as it is missing in the *Hoxa-2* mutant embryos, this region of the otic capsule is likely derived from the second arch neural crest.

The malleus seems to be formed from at least two different primordia. The earliest one, which appears as an en-

largement in the caudal extremity of Meckel's cartilage, generates the body of the ossicle; the second one, which is independent of the first one, generates the manubrium. The independent formation of these two elements is not surprising since, from the analysis of the *gsc* and *Mhox* mutant mice, they seem to be controlled by different genetic mechanisms (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Martin *et al.*, 1995). The neck that connects these two malleal primordia is among the last middle ear elements to be formed. From the embryos I have analyzed, it is not possible to determine whether it originates from an independent primordium or as an extension of any of the previously formed primordia of the malleus. However, the finding that in both the *gsc* and *MHox* mutant mice the manubrium of the malleus was truncated while the body and the neck appeared largely unaffected (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Martin *et al.*, 1995) seems to indicate that at least the neck and the manubrium of the malleus are not ontogenically connected.

It is interesting to note that three of the elements that take part in the morphogenesis of the eardrum (tympanic ring, manubrium of the malleus, and external acoustic meatus) develop at the same time. However, the fourth of the elements, the tubotympanic recess, was absent or very reduced in the vast majority of the embryos that I have analyzed. The absence of this recess, which derives from the first pharyngeal pouch, is very likely a consequence of the fusion of the first and second branchial arches that occurs after RA treatments at this time of development (Goulding and Pratt, 1986; Webster *et al.*, 1986; Pratt *et al.*, 1987; Lee *et al.*, 1995; unpublished results). This would imply that it reaches the anatomical position in which it can become part of the tympanic membrane in a quite passive fashion. This contrasts with the formation of the external acoustic meatus, which is derived from the first pharyngeal cleft (also affected by the first and second arch fusion); it is guided into position by the tympanic ring (Mallo and Gridley, 1996). This view is supported by the finding that in *gsc* mutant embryos, which have normal pharyngeal clefts and pouches, the formation of the tubotympanic recess is not affected, while the tympanic ring and external acoustic meatus fail to develop (Rivera-Pérez *et al.*, 1995). Moreover, in the absence of the tubotympanic recess, the external acoustic meatus, the manubrium of the malleus, and the tympanic ring were able to form a structure clearly resembling a typical eardrum, which also supports the hypothesis that the formation of the lateral and medial surfaces of the eardrum (provided respectively by the external acoustic meatus and tubotympanic recess) is controlled by two different mechanisms.

The stapes seems to develop from two different primordia, one for the footplate and another for the arch, the latter arising later in development. This dual stapedial origin is interesting in light of the effects of the mutations of *MHox* and *Dlx-2* genes on the shape of the stapes (Martin *et al.*, 1995; Qiu *et al.*, 1995). In both cases the footplate seemed to be normal while the two branches of the arch were fused. Thus, the two stapedial primordia might be regulated by independent genetic mechanisms. The correct morphogenesis of the stapes is, however, independent of its contact with the incus since the morphogenesis of this bone is complete before the long process of the incus has developed.

In summary, with RA treatments I have dissected several aspects in the morphogenesis of the ear structures. Further experiments will be required to define how the genesis of those elements and their morphogenetic interactions are controlled and coordinated to generate a functional ear.

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