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# Studies on the morphology of the inner ear and semicircular canal end-organ projections of *ha*, a medaka behavior mutant

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**Abstract** The morphology of the inner ear was investigated in the mutant strain *ha* of medaka (*Oryzias latipes*). The *ha* is a recessive mutant and *ha* homozygotes are viable but show abnormal circular swimming behavior. In adult *ha/ha* medaka, more than one semicircular canals are absent. In the most abnormal cases, no canals are present at all and the membranous labyrinth of the inner ear exhibits a simple rugby-ball-like structure. In spite of the apparent absence of the canals, however, receptor endorgans of the canals (crista ampullaris) and the nerves innervating the cristae (ampullar nerves) are present. Otoliths and associated receptor epithelia (maculae) as well as octaval nerve branches innervating maculae are also present, except utricular otoliths that are absent or extremely small if present. Projections of the ampullar nerves were also investigated, because central connections of the nerves may be also abnormal. Tract-tracing studies, however, revealed similar central projection patterns of primary afferents in the mutant and wild-type brains. These results suggest that membranes of prospective semicircular canals fail to form tubular structures and fuse with the membranes of otolith organs in *ha/ha* medaka. These results also suggest that abnormal morphology of the semicircular canals as well as the utricular otolith underlies the abnormal swimming behavior of the *ha/ha* medaka, in spite of apparently normal central projections of the ampullar nerves.

## Introduction

The inner ear of teleost fishes serves as a sensor for gravity as well as linear and angular accelerations to mediate information indispensable for maintaining appropriate body posture in their watery habitat. There are three otolith organs (utriculus, sacculus, and lagena) and three semicircular canals (anterior, horizontal, and posterior canals) on each side of the head. In general, otolith organs are considered to detect linear acceleration and gravity, and semicircular canals are considered to sense angular accelerations, although acoustic sensory functions of some otolith organs are also known in teleosts (Platt, 1983; Popper, 1983). The sensory epithelium of each otolith organ, known as the macula, lies below the otolith and each semicircular canal contains its own sensory endorgan, the crista ampullaris. The octaval nerve sends branches to innervate each macula (utricular, saccular, and lagenar nerves) and crista ampullaris (anterior, posterior, and horizontal ampullar nerves).

A spontaneous recessive mutant *ha* was found from the orange-red medaka (*Oryzias latipes*) and established by the late Professor Hideo Tomita (1931–1998). Homozygous *ha* medaka are viable and show abnormal morphology in the inner ear and circular swimming behavior. When *ha/ha* medaka are surprised and swim rapidly, they turn around a rostro-caudal axis (Tomita and Takeuchi, unpublished observation referred to in: http://bio.nagoya-u.ac.jp:8000/TomitaH.html). Although the gene responsible for *ha* phenotypes remains to be determined, the *ha* mutant provides a good model to study the functions and development of the vestibular system by comparison with the wild type. Morphological and behavioral abnormalities, however, have only been reported in an abstract form by Tomita (1980). More recent studies on *ha/ha* medaka have reported the behavioral characteristics under microgravity (Ijiri, 2000; Ijiri *et al.*, 2003) and the malformation of otoliths (Mizuno and Ijiri, 2003). However, the morphology of the inner ear, including membranous and bony struc-

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tures (labyrinths), sensory innervations, and central connections, remains to be fully investigated.

In the present study, a detailed morphological analysis of the inner ear in *ha/ha* medaka was performed with complete sets of frontal sections of the head. Sections were immunocytochemically stained with anti-neurofilament antibody to facilitate observations of nervous structures and counterstained with cresyl violet. Central projections of the ampullar nerves were also studied, because neural structures may be also affected in *ha/ha* medaka.

## Materials and Methods

A total of 24 *ha/ha* medaka (21–26 mm in standard length) and 22 normal orange-red medaka (control fish of matching sizes) were used in the present study. The *ha* strain was originally supplied by Professor Yuko Wakamatsu (Nagoya University). The experimental procedures of the present study are in accord with the official Japanese regulations for research on animals and the guidelines of the Animal Care Committee of Nippon Medical School.

### *Morphology of the inner ear*

Thirteen adult *ha/ha* medaka (10 males and 3 females) and 10 adult orange-red medaka (7 males and 3 females) were used for histological examination. The fish were anesthetized by immersion into 3-aminobenzoic acid ethyl ester (MS222; Sigma [St. Louis, USA]) solution (300 mg/l) and perfused through the conus arteriosus with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The heads of selected fish were observed for examination of external morphology under a dissection microscope and photographed prior to histological procedures described below. A portion of each cranium was opened and the heads were post-fixed in a fresh solution of the same fixative for 12 hours at 4°C. The heads were then decalcified by immersion into 5% ethylenediamine tetraacetic acid (EDTA) for 24 hours, cryo-protected by immersion in 0.1M phosphate buffer containing 20% sucrose for 24 hours, and embedded in 5% agar (Type IX, Sigma) containing 20% sucrose. The blocks were frozen by immersion into –60°C n-hexane and 40-μm thick frontal sections were cut on a cryostat. Thaw-mounted sections on gelatin-coated slides were dried by fanning for 60 min.

Following two 10-min washes with 0.05 M phosphate-buffered saline (PBS, pH 7.4) and one 10-min wash with PBS containing 0.3% Triton X-100 (PBST), sections were reacted with anti-neurofilament antibody (anti-neurofilament [70k+160k+210k]; Cosmo Bio, Tokyo, Japan) diluted to 1:1000 in PBST overnight. This antibody has been used in medaka and is known to label peripheral nerves (Ishikawa *et al.*, 1986; Ishikawa and Iwamatsu, 1993). To reduce non-specific reactions, normal horse serum was added to the primary antiserum solution to a final concentration of 1%. Sections were then rinsed by three 10-min washes in PBS and reacted for 1h with secondary antiserum solution (anti-mouse IgG [Vector, Burlingame, USA] diluted to 1:200 in PBST). After the reaction with secondary antiserum, sections were treated with 0.3% $H_2O_2$ solution in methanol to block endogenous peroxidase activities and incubated with avidine-biotin-complex solution (1:50; Sigma, ABC elite kit) for 1.5 h at room temperature. After three 10-min washes in PBS, the sections were reacted with diaminobenzidine (DAB) after the protocol of Adams (1981), counterstained with 0.025% cresyl violet, dehydrated, and placed under coverslips. All of the reactions were carried out at room temperature. Stained serial sections were observed to examine the morphology of the bony labyrinth, membranous labyrinth (semicircular canals and associated crista ampullaris as well as otolith organs and the associated otolith and macula), and the innervation pattern of the sensory epithelium.

### *Tract-tracing studies of ampullar nerves*

Pilot experiments performed *in vivo* resulted in high mortalities of fish due to severe damage from opening the cranium to expose the inner ear. Therefore, we performed tract-tracing experiments *in vitro* after procedures developed by Yamamoto *et al.* (1998).

Eleven adult *ha/ha* medaka (5 males and 6 females) and 12 adult orange-red medaka (5 males and 5 females) were deeply anesthetized in MS222 solution (over 300 mg/l). The fish were immersed in Krebs-Ringer's solution aerated with a gas mixture (95% $O_2$ and 5% $CO_2$) and the cranium was opened to expose the crista ampullaris and ampullar nerve. The solution was aspirated when biocytin (Sigma) crystals were applied to the nerve with an insect pin. After decapitation, the head was kept for 4 h in the aerated solution with approximately 75 mg/l MS222. Other procedures were performed according to those of Yamamoto *et al.* (1998).

After 4 h, each head was fixed in 4% paraformaldehyde and processed as described above to prepare 40-μm frontal sections. Then, sections were reacted with avidine-biotin solution (1:100) in PBST overnight at room temperature. Visualization of the tracer and counterstaining were performed as described above for histological examinations of the inner ear. Nuclear identification of the octavolateral area in the medulla oblongata was based on McCormick (1983) and Ishikawa *et al.* (1999).

## Results

### *External morphology*

The *ha/ha* medaka can be discriminated from normal orange-red medaka from the external appearance. In *ha/ha* medaka, the temporal portions of the heads, where the inner ear is embedded, are clearly larger than those in normal orange-red medaka (Fig. 1). Such an external appearance reflects expanded membranous and bony labyrinths in *ha/ha* medaka as described below in detail.

### *Membranous labyrinth*

1. Semicircular canals

In all the *ha/ha* medaka examined, more than one semicircular canals were absent (Table 1). In the extreme cases (n=2 fish), no canals were present (Figs. 2, 3). In such cases, the membranous labyrinth was huge and exhibited a simple rugby-ball-like structure (Fig. 2). Absence of the anterior and posterior canals was encountered very frequently (roughly 90%), while the absence of the horizontal canals was noted less frequently (roughly 40%; Table 2). As expected, in normal orange-red medaka, all three canals were present on both sides in all fish examined (n=10 fish; Figs. 2, 3).

**Table 1**. Number of missing semicircular canals in *ha/ha* medaka.

| number of missing canals | 6 | 5 | 4 | 3 | 2 | 1 | 0 |
|---|---|---|---|---|---|---|---|
| number of fish | 2 | 5 | 2 | 3 | 1 | 0 | 0 |

**Table 2**. The ratio of missing semicircular canals in *ha/ha* medaka.

| anterior canal | horizontal canal | posterior canal |
|---|---|---|
| 23 / 26 sides | 10 / 26 sides | 23 / 26 sides |

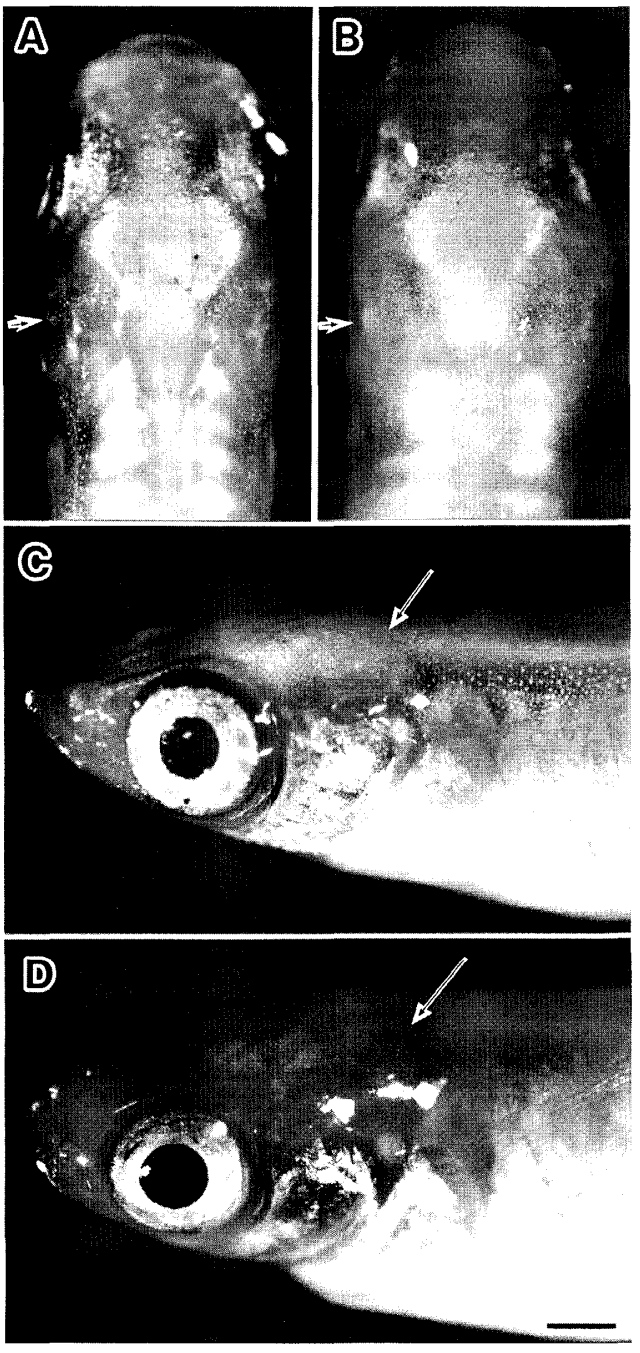



**Fig. 1** External morphology of normal (A, C) and *ha/ha* (B, D) medaka. A-B) Dorsal view. The temporal region of the head where the inner ear is present is indicated by an arrow. This region appears swollen in *ha/ha* medaka. C-D) Lateral view. Scale bar in D (= 1 mm) applies to all panels.

When some canals were present in *ha/ha* medaka, existing canals tended to be short as compared with those in normal fish. Sometimes posterior canals were as short as 80 μm (Fig. 4), whereas in normal orange-red medaka, posterior canals were 300 μm or longer. Absence of canals occurred with almost equal probabilities on both sides (Table 3). However, the lack of canals did not always happen in symmetrical patterns (Fig. 4).

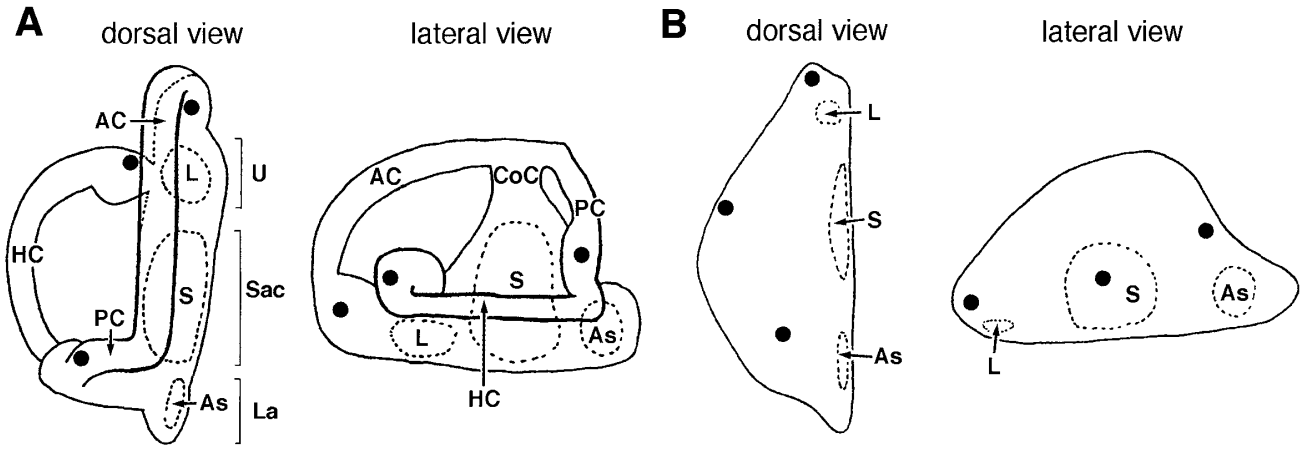
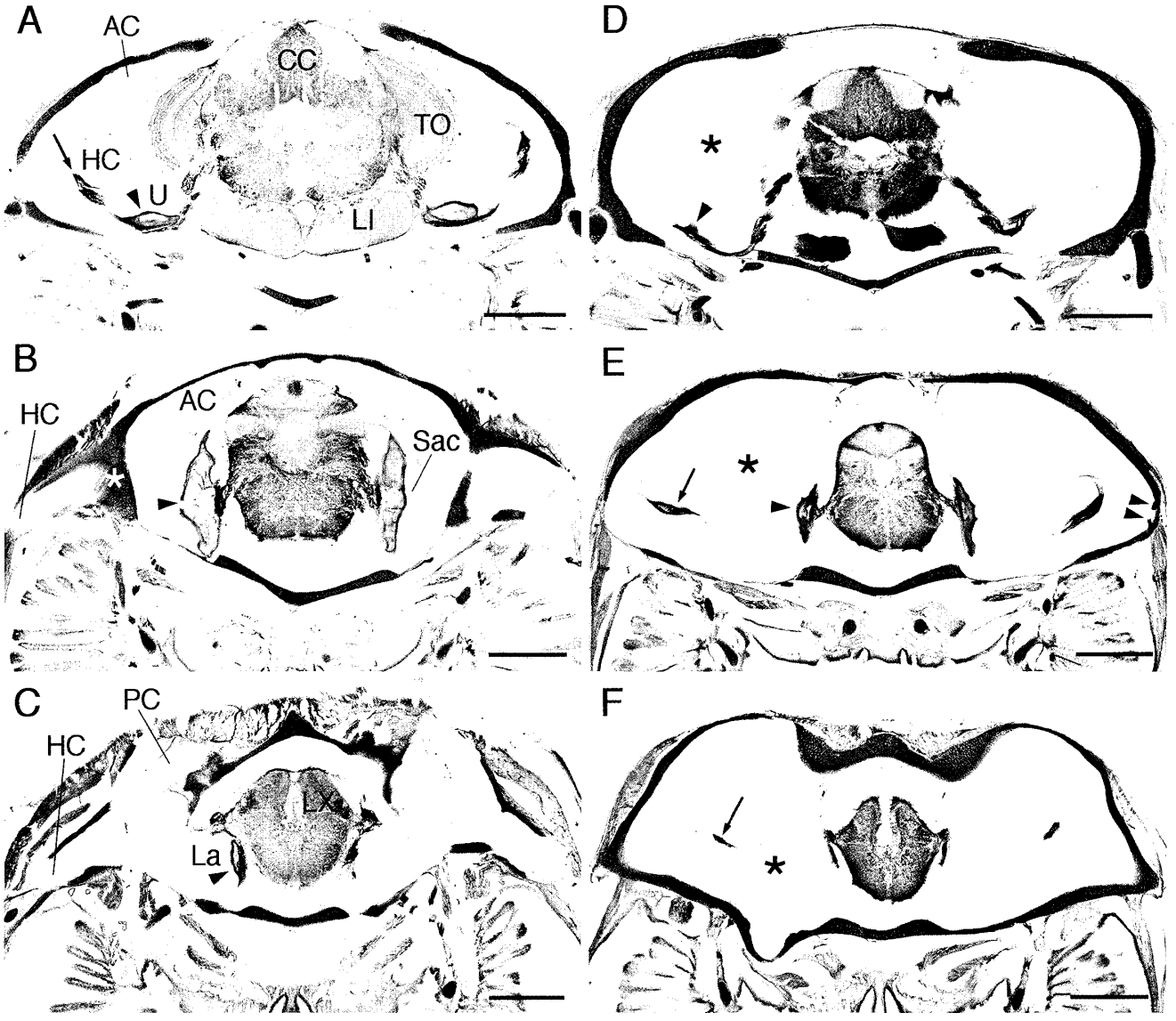



**Fig. 2** Schematic drawings of the membranous labyrinth in normal orange-red medaka (A) and *ha/ha* medaka with no apparent canals (B). Black dots indicate the cristae ampullarum of the semicircular canals. Structures quite similar to the cristae ampullarum of normal fish were present in the inner ear of *ha/ha* medaka, and they are regarded as cristae although canals were not present (see text for further explanation). AC: anterior canal; As: astericus (otolith of lagena); CoC: common crus; HC: horizontal canal; L: lapillus (otolith of utriculus); La: lagena; PC: posterior canal; S: sagitta (otolith of sacculus); Sac: sacculus; U: utriculus. Rostral is to the top for dorsal views and to the left for lateral views. Lateral is to the left for dorsal views and dorsal is to the top for lateral views.



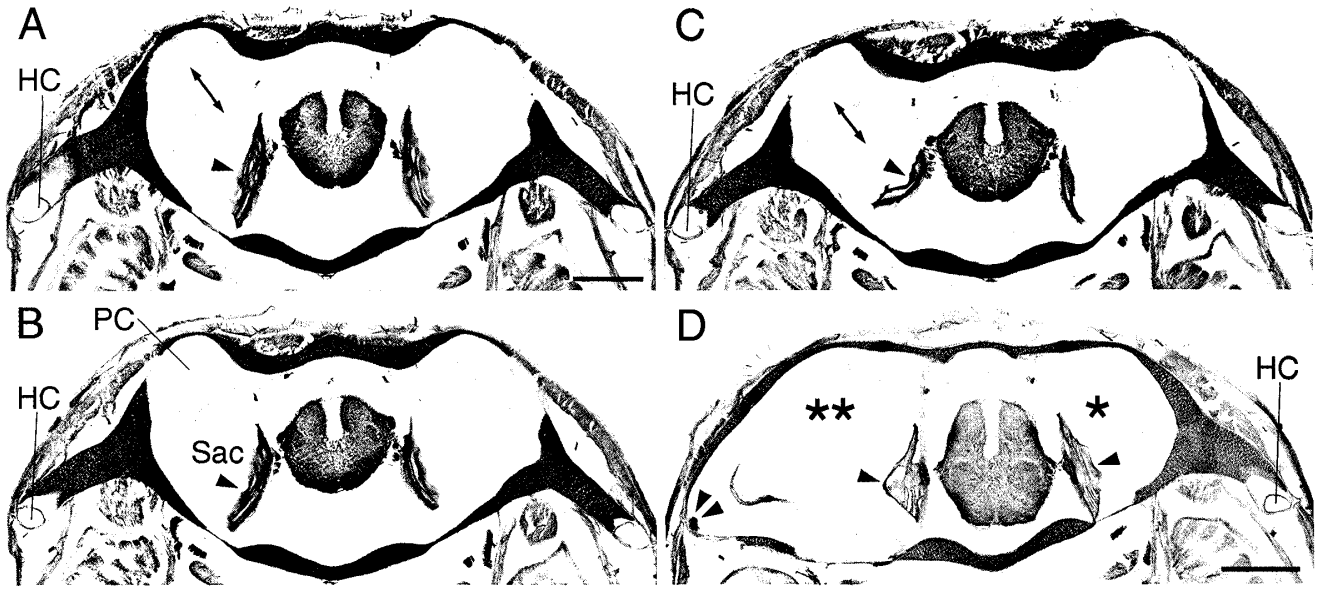



**Fig. 4** Asymmetry of the semicircular canals in *ha/ha* medaka. A *ha/ha* medaka with a tiny posterior canal present only on the right side (A-C) and another *ha* medaka with a horizontal semicircular canal present only on the left side (D) are shown. A-C) The posterior canal of the right side is separate from the membranous labyrinth proper in the section shown in B. However, the canal is connected to the membranous labyrinth proper in the rostrally adjacent section shown in A and in the section shown in C (second next section caudal to B). That is, the canal is present only in two sections, indicating that the canal is about 80 μm rostrocaudally. The crista ampullaris of this posterior canal was present in the membrane of otolith organ, but not in the canal. The left posterior canal was missing. An arrowhead indicates the otolith of the sacculus (or sagitta). D) The horizontal canal is present on the right side, but not on the left side in this *ha/ha* medaka. On the left side is a large endolymphatic space formed by the fusion of the membranes of the posterior canal and sacculus (asterisk), and on the right side is a gigantic space formed by the fusion of the membranes of the horizontal canal, posterior canal, and sacculus (double asterisks). An arrowhead indicates the otolith of sacculus (or sagitta). A bony structure is present on the right side (double arrowheads), which presumably is the remnant of a bone that would separate the horizontal canal from the otolith membrane in normal fish. HC: horizontal canal; PC: posterior canal; Sac: sacculus. Scale bar = 500 μm (A-D). The bar in A applies to B and C.

**Fig. 3** Frontal sections of normal orange-red medaka (A-C) and *ha/ha* medaka with no semicircular canals (D-F). A) Section through the caudal margin of the optic tectum. An arrow indicates the crista ampullaris of the horizontal canal. An arrowhead indicates the utricular otolith (or lapillus) and underlying macula. The anterior canal is also visible. B) Section at the caudal margin of the cerebellum. The sacculus, anterior canal, and horizontal canal are visible. The horizontal canal is separated from other parts of the membranous labyrinth by the bony labyrinth (white asterisk). An arrowhead indicates the otolith of sacculus (or sagitta). C) Section at the level of the vagal lobe. Horizontal and posterior canals as well as the lagena are visible. An arrowhead indicates the rostral margin of the otolith of the lagena (or astricus). D) Section that corresponds roughly to the level shown in A. No canal is present and the membranous labyrinth forms a single, large endolymphatic space (asterisk). An arrowhead indicates an extremely small otolith of the utriculus (lapillus) and underlying macula. On the contralateral side was a lapillus with a size comparable to the ones in normal fish caudal to sections shown in this panel. Also note the expanded space surrounded by bones (or bony labyrinth). E) Section that corresponds roughly to the level shown in B. No canal is present and the membranous labyrinth forms a single, large endolymphatic space (asterisk) also at this level. An arrowhead indicates the saccular otolith (sagitta) that is smaller and thinner than those in normal medaka. An arrow indicates a sensory epithelium that appears to correspond to that in the crista ampullaris of the horizontal canal. Note the caudal location compared with that in normal medaka. Tiny bony structures are present (double arrowheads) that appear to correspond to the bone separating the horizontal canal from other membranous labyrinths in normal fish (B: white asterisk). F) Section that corresponds roughly to the level shown in C. No canal is present and the membranous labyrinth forms a single, large endolymphatic space (asterisk) also at this caudal level. An arrow indicates the sensory epithelium and nerve fibers innervating it, corresponding to the crista ampullaris of the posterior canal. AC: anterior canal; CC: corpus cerebelli; HC: horizontal canal; La: lagena; LI: lobus inferior; LX: vagal lobe; PC: posterior canal; Sac: sacculus; TO: tectum opticum; U: utriculus. Scale bar = 500 μm (A-F).

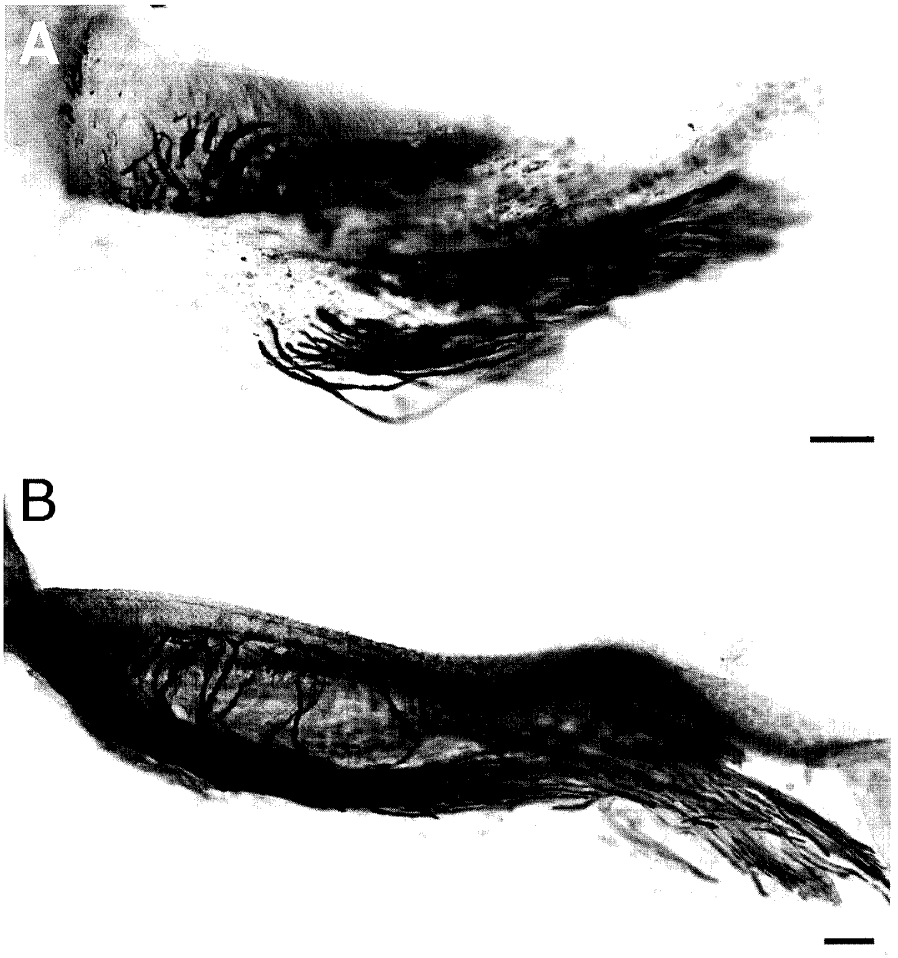



**Fig. 5** Sensory epithelia of the cristae ampullarum of the horizontal semicircular canals of normal orange-red (A) and *ha/ha* (B) medaka. Fibers of the horizontal ampullar nerve innervating the sensory epithelium are visible. Scale bar = 50 μm.

**Table 3.** The ratio of missing semicircular canals in the left and right sides in *ha/ha* medaka.

| | anterior canal | horizontal canal | posterior canal |
|---|---|---|---|
| left | 11 / 13 sides | 4 / 13 sides | 12 / 13 sides |
| right | 12 / 13 sides | 6 / 13 sides | 11 / 13 sides |

2. Crista ampullaris of the semicircular canals

In *ha/ha* medaka that possessed only some of the semicircular canals, cristae ampullarum for the existing canals were present. When the existing canals were not extremely short, their cristae were located in almost normal positions. However, cristae of remarkably short canals were not located within the canals but on the membranous labyrinth of the otolith organ to which the canal was connected. In addition, cristae of horizontal canals were located more caudally compared with normal fish in many cases (Fig. 3). Cristae of anterior or posterior canals were also located at abnormal rostrocaudal levels in some cases.

In *ha/ha* medaka with no canals at all, three sensory epithelia were found on both sides, in addition to three pairs of maculae of otolith organs (Figs. 2, 3). Also, the morphology of these sensory epithelia was quite similar to that of the sensory epithelium of the crista ampullaris of normal medaka (Fig. 5). These sensory epithelia, therefore, may be regarded as those of the anterior, horizontal, and posterior canals in this order from rostral to caudal.

3. Otolith organs

In all *ha/ha* medaka examined, all of the three otolith organs (utriculus, sacculus, and lagena) were present. The macula of each otolith organ was present and located in a roughly similar position to that in normal orange-red medaka, and no morphological abnormalities were detected as compared with normal orange-red medaka examined in the present study.

Some of the *ha/ha* medaka lacked otoliths of the utriculus (12 /26 sides). The lack of utricular

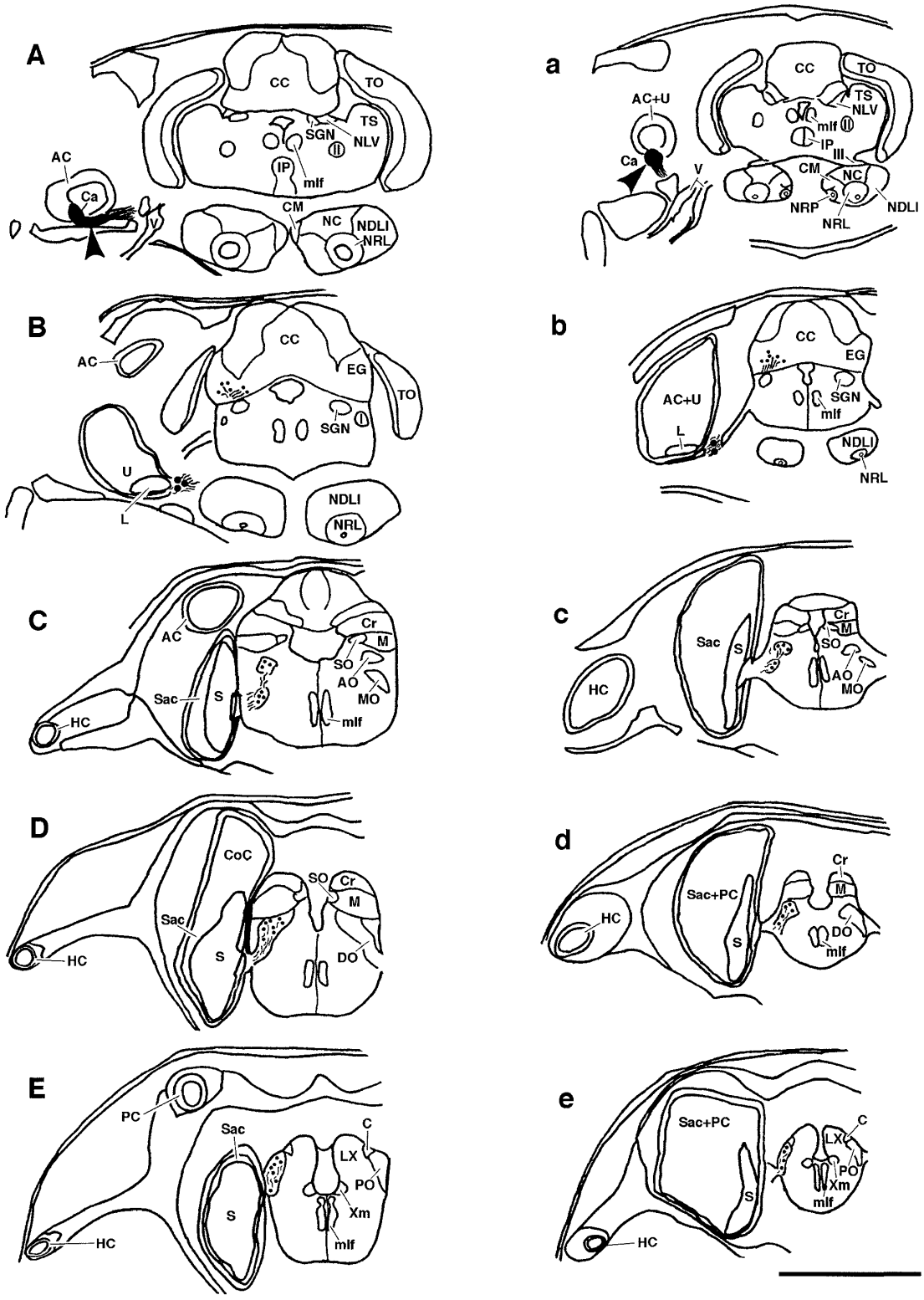



**Fig. 6** Line drawings illustrating structures labeled by biocytin applications *in vitro* into the anterior ampullar nerve of normal orange-red (A-E) and *ha/ha* (a-e) medaka. Arrowheads in A and *a* indicate the application sites of biocytin. Large dots indicate labeled ganglion cells of the ampullar nerve, lines indicate labeled fibers, and small dots indicate labeled axon terminals. AC: anterior canal; AO: anterior octaval nucleus; C: caudal nucleus of the rhombencephalic octavolateral area; Ca: crista ampullaris of anterior canal; CC: corpus cerebelli; CM: corpus mamillare; CoC: common crus; Cr: crista cerebellaris; DO: descending octaval nucleus; EG: eminentia granularis; HC: horizontal canal; I: nucleus isthmi; IP: nucleus interpeduncluraris; L: lapillus; ll: lateral lemniscus; LX: vagal lobe; mlf: medial longitudinal fasciculus; M: nucleus medialis of the rhombencephalic octavolateralis area; MO: magnocellular octaval nucleus; NC: nucleus centralis lobi inferioris; NDLI: nucleus diffusus lobi inferioris; NLV: nucleus lateralis valvulae; NRL: nucleus recessi lateralis; NRP: nucleus recessi posterior; PC: posterior canal; PO: posterior octaval nucleus; S: sagitta; Sac: sacculus; SGN: secondary gustatory nucleus; SO: secondary octaval nucleus; TO: tectum opticum; TS: torus semicircularis; U: utriculus; Xm: dorsal motor nucleus of vagus; III: oculomotor nerve; V: trigeminal nerve. Scale bar = 1 mm.

otolith occurred in 5 left sides and 7 right sides, suggesting absence of laterality in the expression of the abnormal phenotype. Some of the existing utricular otoliths were very tiny (Fig. 3D). Saccular and lagenar otoliths were present in all fish. However, saccular otoliths were remarkably small in many *ha/ha* medaka compared with normal orange-red fish, compressed mediolaterally (Figs. 3E, 4). No morphological abnormalities were detected in the lagenar otoliths surveyed in the present study.

### *Bony labyrinth*

In all *ha/ha* medaka examined, expansion of the bony labyrinth was noticeable parallel to the gigantic membranous labyrinth. This tendency was especially marked in *ha/ha* medaka with severe abnormality in the membranous labyrinth (Figs. 3, 4D).

In normal orange-red medaka, bony structures were present that separated the otolith membrane from the horizontal canal membrane (Fig. 3B). In contrast, in *ha/ha* medaka without horizontal canals, such bony structures were absent. Instead, tiny bones were observed outside the membranous labyrinth (Fig. 3E), which most likely represent remnants of the bones separating the horizontal canal from the otolith membrane.

### *Innervation of the inner ear*

In *ha/ha* medaka, the innervation pattern of the octaval nerve may be regarded to be the same as in normal medaka in that the nerve innervates three pairs of maculae and cristae. However, abnormal courses of the horizontal ampullar nerve were noted. In normal orange-red medaka, the horizontal ampullar nerve ran ventrally adjacent to the utricular nerve, while in *ha/ha* medaka, the nerve ran some distance from the utricular nerve. This abnormal course might be related to the posterior shift of the crista of the horizontal semicircular canal, compared with normal fish. In one case, the horizontal ampullar nerve was running through the bone, which was never seen in normal orange-red medaka.

### *Central connections of ampullar nerves*

Biocytin applications to the anterior ampullar nerve in normal orange-red medaka resulted in labeled terminals in the eminentia granularis, anterior octaval nucleus, magnocellular octaval nucleus, descending octaval nucleus, and posterior octaval nucleus ipsilateral to the side of tracer applications (Fig. 6A-E). Biocytin applications to the posterior ampullar nerve resulted in labeled terminals in the same nuclei as those seen after anterior semicircular experiments (data not shown). Biocytin applications to the horizontal ampullar nerve resulted in labeled terminals in the nucleus tangentialis in addition to those nuclei that receive fibers from anterior and posterior canals (data not shown). Labeled fibers in the eminentia granularis, however, were only one or two. Central connections of the ampullar nerves in normal orange-red medaka are thus quite similar to those known in other teleosts (Meredith and Butler, 1983; McCormick and Braford, 1993, 1994).

Biocytin applications to the anterior, posterior, and horizontal ampullar nerves in *ha/ha* medaka resulted in labeled terminals in the same nuclei as in normal orange-red medaka with much the same distribution pattern in each target nucleus (Fig. 6a-e: anterior ampullar nerve injection).

### *Brain size*

Although we did not analyze quantitatively in the present study, the brain in *ha/ha* medaka appeared much smaller than in normal orange-red medaka (Fig. 3).

## Discussion

So far as we know, this is the first report that investigated fully the morphology of the inner ear of *ha/ha* medaka from the bony labyrinth to the membranous labyrinth including maculae, otoliths, and cristae ampullarum. Furthermore, the present study also examined the morphology of the octaval nerve with a special focus on the central connections of the ampullar nerves.

In the present study, the membranous labyrinth of *ha/ha* medaka was found to show remarkable abnormalities, especially regarding the semicircular canals. This is in basic agreement with a previous study reporting the total absence of semicircular canals (Tomita, 1980; also see unpublished observation by Tomita and Takeuchi that is presented in a website: http://bio.nagoya-u.ac.jp:8000/TomitaH.html). An extreme phenotype was also observed in the present study. However, we also encountered other phenotypes in *ha/ha* medaka, in which one to four semicircular canals were present. Also importantly, all cristae ampullarum and ampullar nerves innervating them were always present in *ha/ha* medaka, irrespective of the degree of malformation of canals. The cristae were present within the canals when the malfor-

mation was modest. When the malformation was severe, cristae were present on the membrane that apparently seemed to be the otolith organ membrane. These findings suggest that the membrane of semicircular canals fails to form a tubular configuration and becomes fused with or absorbed by the membrane of otolith organs. We consider that variability in the extent of fusion is reflected in the variety of morphology of the membranous labyrinth observed in the present study.

The morphology of semicircular canals was not always symmetrical in the *ha/ha* medaka studied in the present study. However, the absence of canals did not occur with a bias to the left or right side. These results suggest that semicircular canals of *ha/ha* medaka become absent with the same probability on both sides in an independent manner. The present study also revealed that the horizontal canal becomes absent with a low probability (about 40%), in sharp contrast to the anterior and posterior canals that are missing in about 90% of the sides examined in the present study. This may be related to the presence of a bone that separates the horizontal canal from the membrane of otolith organs. Fusion of the horizontal canal may occur only in some *ha/ha* medaka in which the bone fails to attain normal morphology. Similar bony structures that separate the anterior and posterior canals from the membrane of otolith organs are not present and fusion may occur more easily. The cause of such fusion remains unknown. One possible explanation is the excessive production or defective drainage of endolymph in *ha/ha* medaka, like Menier's disease in humans. Hyperpressure of endolymph may expand the membrane labyrinth to hinder the formation of tubular membranous structures and may also expand the bony labyrinth, because *ha* phenotypes start in embryos prior to ossification.

Semicircular canals are tubular structures that protrude from the otolith organ membrane, arranged three-dimensionally to detect angular acceleration or rotation in any direction. Rotation of the head is detected by the cilia of hair cells that are deflected by the relative (inertial) movement of endolymph against the crista. Circular configuration of the canals, therefore, is a crucial feature for efficient and accurate detection of angular acceleration. In *ha/ha* medaka, more than one semicircular canals are absent and those canals present are severely deformed. That is, "semicircular" canals may be said to be almost completely lacking in this mutant medaka, in particular from the functional point of view. Therefore, detection of angular acceleration or rotation must be seriously defective in *ha/ha* medaka, even though sensory epithelia and respective nerves innervating them are present. Tract-tracing experiments of the present study suggest that ampullar nerves of *ha* medaka send information to the same targets and with similar terminal patterns as in normal orange-red medaka. Thus, defective sensory information from the semicircular canals does not appear to be compensated by the reorganization of the central connections of ampullar nerves examined morphologically in the present study. The above-mentioned morphological features of semicircular canals thus appear to underlie the abnormal circular swimming characteristic in *ha/ha* medaka.

Otolith organs detect linear acceleration involving gravity as well as acoustic stimuli (Popper, 1983; Platt, 1983). It is considered that gravity is sensed by the utriculus, although involvement of the sacculus is also suggested (Popper, 1983; Platt, 1983). In *ha/ha* medaka studied in the present study, utricular otoliths (or lapillus) were absent or very small if present, in agreement with a previous study (Mizuno and Ijiri, 2003). Therefore, detection of linear acceleration involving gravity by the utricular macula must be severely defective. It was also found in the present study that many saccular otoliths (or sagitta) are small and thin, compressed mediolaterally. The present study, therefore, suggests that abnormalities in utricular and possibly saccular otoliths also contribute to the abnormal swimming of this mutant strain. The sacculus is known to be involved in auditory functions as well, in particular in otophysans (Popper, 1983). Possible defects in the acoustic sensory ability in *ha/ha* medaka due to abnormal saccular otoliths remain to be examined.

Recently, Maruyama *et al.* (2000; 2002; 2005) identified otolith matrix proteins in salmonids (OMP-1 and Otolin-1) and zebrafish (zOMP-1 and zOtolin-1). Injections of antisense morpholino-oligonucleotides (MOs) to knockdown *zomp-1* and *zotolin-1* during development resulted in abnormal morphology of the inner ear that is similar to *ha/ha* phenotypes (Maruyama *et al.*, 2005). The otoliths observed in *zomp*-1 MO-injected zebrafish were smaller than those in normal fish, a situation similar to the utricular and saccular otoliths in *ha/ha* medaka. The *zotolin-1* MO injections resulted in the fusion of otoliths, which is reminiscent of the situation observed in *ha/ha* embryonic

medaka (Mizuno and Ijiri, 2003). Also importantly, some of the semicircular canals were lacking after injections of either MO. However, there are differences as well. The *zomp*-1 MO injections were more effective in reducing the size of saccular otoliths than that of utricular otoliths, a pattern contrary to that seen in *ha/ha* medaka. Injections with MO resulted in loss of horizontal canals but partial suppression of anterior and posterior canal formation, again a pattern contrary to *ha/ha* phenotypes. Finally, otic vesicles were smaller after MO injections, which appears contrary to *ha/ha* medaka, in which inner ears are larger than in normal fish. The MO-induced phenotypes might not be directly comparable to those of this spontaneous mutant *ha*, and the gene responsible for *ha* phenotypes should be studied further with *omp-1* and *otolin-1* as possible candidates.

Finally, the brain of *ha/ha* medaka appears smaller than that in normal medaka. Further studies on the brain, in particular quantitative analyses in the vestibular-related centers, are important to understand fully the *ha* strain, which is a promising mutant model for understanding the physiology of the inner ear and its developmental mechanisms in comparison with normal fish.

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