Partial Requirement of Endothelin Receptor B in Spiral Ganglion Neurons for Postnatal Development of Hearing*□S

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Impairments of endothelin receptor B (Ednrb/EDNRB) cause the development of Waardenburg-Shah syndrome with congenital hearing loss, hypopigmentation, and megacolon disease in mice and humans. Hearing loss in Waardenburg-Shah syndrome has been thought to be caused by an Ednrb-mediated congenital defect of melanocytes in the stria vascularis (SV) of inner ears. Here we show that Ednrb expressed in spiral ganglion neurons (SGNs) in inner ears is required for postnatal development of hearing in mice. Ednrb protein was expressed in SGNs from WT mice on postnatal day 19 (P19), whereas it was undetectable in SGNs from WT mice on P3. Correspondingly, *Ednrb* homozygously deleted mice (*Ednrb*$^{-/-}$ mice) with congenital hearing loss showed degeneration of SGNs on P19 but not on P3. The congenital hearing loss involving neurodegeneration of SGNs as well as megacolon disease in *Ednrb*$^{-/-}$ mice were markedly improved by introducing an *Ednrb* transgene under control of the dopamine β-hydroxylase promoter (*Ednrb*$^{-/-}$;*DBH-Ednrb* mice) on P19. Neither defects of melanocytes nor hypopigmentation in the SV and skin in *Ednrb*$^{-/-}$ mice was rescued in the *Ednrb*$^{-/-}$;*DBH-Ednrb* mice. Thus, the results of this study indicate a novel role of Ednrb expressed in SGNs distinct from that in melanocytes in the SV contributing partially to postnatal hearing development.

Waardenburg syndrome (WS)[3] involves hearing loss and hypopigmentation. The incidence of WS is 1/10,000–20,000 people (1). Waardenburg-Shah syndrome (WS type IV, WS-IV), caused by mutations of the transcription factor Sox10 (2), the cytokine endothelin (ET)-3 (3) and its receptor endothelin receptor B (Ednrb) (4), is characterized by hypopigmentation, megacolon disease and hearing loss.

Endothelin receptor B (Ednrb/EDNRB) belongs to the G protein-coupled receptor family, mediating pleiotropic actions of endothelins (5, 6). ET-1, ET-2, and ET-3 are ligands for Ednrb with equal affinity (6, 7). Impairments of Ednrb/EDNRB have been shown to cause embryonic defects of melanocytes and enteric ganglion neurons derived from the neural crest, resulting in hypopigmentation, megacolon disease, and congenital hearing loss. In rodent models, piebald-lethal rats in which *Ednrb* is spontaneously mutated (8) and Ednrb homozygous knock-out (*Ednrb*$^{-/-}$) mice (9) have been shown to have typical WS-IV phenotypes. Thus, these previous studies indicate that Ednrb is one of the key regulatory molecules for embryonic development of melanocytes and peripheral neurons including neurons in the enteric nervous system.

The inner ears contain the organ of Corti and stria vascularis (SV). The organ of Corti, which contains two kinds of sensory cells (inner hair cells and outer hair cells), is responsible for mechanotransduction, by which sound impulses are converted into neural impulses. Auditory information from the sensory cells is transmitted to spiral ganglion neurons (SGNs) as the primary sensory carrier for the auditory system, followed by eventual transmission to the auditory cortex (10, 11). Impairments of SGNs have been shown to cause hearing loss (12). Our recent study has also shown that c-Ret-mediated degeneration of SGNs directly causes severe congenital hearing loss (13). The SV consists of marginal cells, melanocytes (also known as intermediate cells), and basal cells and has been shown to maintain high levels of potassium ion for endocochlear potential (EP)

(14, 15). Melanocytes in the inner ear are specifically located in the SV, and these defects lead to impaired EP levels resulting in hearing loss (16). Thus, disturbance of these constituent cells in inner ears has been shown to cause hearing losses (10).

Dopamine β-hydroxylase (DBH) is an enzyme that converts the neurotransmitter dopamine to noradrenaline. DBH has been using as a specific marker of noradrenergic/adrenergic neurons because noradrenaline converted by DBH is secreted as a neurotransmitter from noradrenergic/adrenergic neurons. *DBH* promoter has been used as a valuable tool to allow a target gene to be expressed in peripheral neurons derived from the neural crest *in vivo* (17). A previous study showed that aganglionic megacolon disease in *Ednrb* homozygously deleted mice (*Ednrb$^{-/-}$* mice) was recovered by the introduction of an *Ednrb* transgene driven by the human *DBH* promoter (*Ednrb$^{-/-}$*; *DBH-Ednrb* mice) (18). However, there is no information about hearing levels in *Ednrb$^{-/-}$;DBH-Ednrb* mice. Previous studies have shown that endogenous DBH is expressed in SGNs of inner ears (19), whereas neither endogenous DBH nor a transgene driven by the *DBH* promoter is expressed in melanocytes or their precursors (17, 20). Thus, these results of previous studies suggest that the *DBH* promoter enables Ednrb protein to be specifically expressed in SGNs.

Previous studies demonstrated that impairments of Ednrb/EDNRB cause hypopigmentation and megacolon disease due to defects of melanocytes and peripheral neurons such as enteric ganglion neurons, respectively (4–6, 8, 9). There has been only one report showing that Ednrb-mediated hearing loss involved a congenital defect of melanocytes in the SV (9). However, there was no information in that report about the role of Ednrb in SGNs, which serve as peripheral neurons in inner ears for the auditory system, although it was shown in the present study that Ednrb protein is expressed in SGNs. The results of the present study indicate a novel etiology for Ednrb-mediated hearing loss in mice that can involve postnatal degeneration of SGNs besides congenital defects of melanocytes in the SV.

EXPERIMENTAL PROCEDURES

Mice—*Ednrb$^{-/-}$* mice (5) and *Ednrb$^{-/-}$;DBH-Ednrb* mice (18) were reported previously. All experiments were authorized by the Institutional Animal Care and Use Committee in Chubu University (approval number 2110017) and the Institutional Recombinant DNA Experiment Committee in Chubu University (approval number 06-01) and followed the Japanese Government Regulations for Animal Experiments.

Measurement of Hearing—Tone burst-evoked auditory brainstem response (ABR) measurements (AD Instruments Pty. Ltd.) were performed as described previously (13, 21). Tone burst stimuli were measured 5 dB stepwise from 0 decibel sound pressure level (dB SPL) to 70 or 90 dB SPL. The threshold was obtained by identifying the lowest level of the I wave of ABR recognized. Data are presented as means ± S.E.

Morphological Analysis with a Light Microscope—After perfusion fixation by Bouin's solution, cochleae from postnatal day 19 (P19) mice were immersed in the same solution for 1 week. H&E staining and immunohistochemical analyses with anti-Ednrb (1:2000; Chemicon) and anti-Kir4.1 (1:500; Santa Cruz Biotechnology) antibodies were performed with paraffin sec-

tions. The VECTASTAIN ABC kit (Vector) and Envision kit/HRP (diaminobenzidine; DAKO) were used in the immunohistochemical analyses with a hematoxylin counterstain. In the case of anti-Kir4.1, the Vector VIP substrate kit for peroxidase (Vector) was used with counterstained methyl green. LacZ staining of dopachrome tautomerase (*Dct*)-*LacZ* melanocytes was performed as described previously (13). In brief, after fixation with PBS containing 0.25% glutaraldehyde, the inner ears were stained with 0.04% X-Gal by intracochlear perfusion. The samples were postfixed with 4% paraformaldehyde and decalcified with EDTA, and then paraffin sections were prepared. Estimation of cell density of SGNs with H&E staining basically followed the previous method (13, 22–24). In brief, the area of Rosenthal's canal in three sections from each mouse was measured with the software program WinROOF (version 6.2; Mitani Corp., Fukui, Japan) as reported previously (13, 24). Cell density of SGNs from three mice for each mouse strain was calculated by dividing the cell number of SGNs in the measured Rosenthal's canal by the area of the section examined. A total of 100–150 SGNs in three sections from each mouse were examined. Percentage of positive signals histochemically detected by antibodies or LacZ staining was estimated with WinROOF (version 6.2) as reported previously (13, 24). Briefly, the number of positive SGNs was divided by the total number of SGNs. A total of 100–150 SGNs in five sections from each mouse were examined. In the case of SV, positive signals in the measured SV were divided by the area of the section measured. To compare the positive levels among mouse strains, the normalized positive signals in *Ednrb$^{-/-}$* mice and *Ednrb$^{-/-}$;DBH-Ednrb mice* were divided by the normalized positive signals in WT mice. Rosenthal's canal or SV from three or four mice for each mouse strain was measured for each estimation.

Morphological Analysis by Transmission Electron Microscopy (TEM)—Preparation of tissues for TEM basically followed the previous method (13, 22). In brief, after perfusion fixation with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.3 M HEPES (pH 7.4), dissected murine cochleae were immersed in the same fixative solution overnight at 4 °C. The cochleae were then fixed with 2% osmium tetroxide in 0.3 M HEPES (pH 7.4) at 4 °C for 3 h. After rinsing off the fixative solution, the cochleae were dehydrated with a graded series of ethanol and embedded in epoxy resin (Quetol 651). Ultrathin sections (t = 70 nm) were observed under an electron microscope at 80 kV (JEOL JEM1200EX, Tokyo, Japan). Additional procedures are described in the supplemental Methods.

Statistics—Significant difference (*, $p < 0.01$; **, $p < 0.05$) from the control was analyzed by the Mann-Whitney *U* test.

RESULTS

Congenital Deafness in Ednrb$^{-/-}$ Mice and Tissue Distribution of Ednrb Protein in Inner Ears—We first measured hearing levels of *Ednrb$^{-/-}$* mice (5) and littermate WT mice on P19 (Fig. 1, *A–C*). ABR thresholds for 4–32-kHz sound in *Ednrb$^{-/-}$* mice (90–95 dB SPL) were much higher than those in littermate WT mice (20–55 dB SPL) (Fig. 1*A*). This result indicates that the *Ednrb$^{-/-}$* mice suffer from severe congenital hearing loss. Immunohistochemical analyses of inner ears showed expression of Ednrb protein in SGNs (*arrow* in Fig. 1*D*) and the
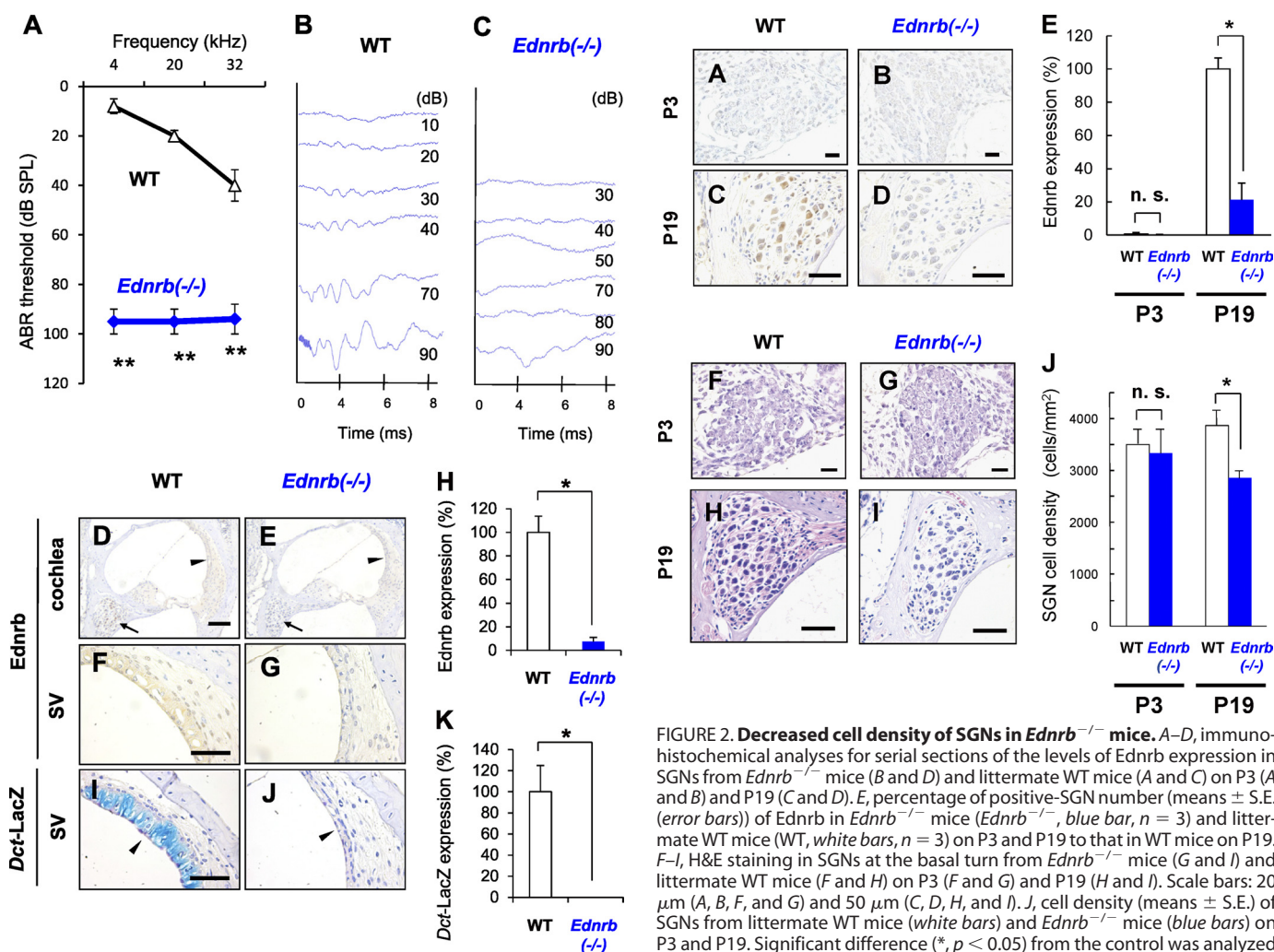
FIGURE 1. Congenital deafness in *Ednrb*^{−/−} mice and expression of Ednrb in inner ears. *A*, hearing levels (means ± S.E. (*error bars*)) in WT mice (*n* = 9) and *Ednrb*^{−/−} mice (*n* = 9) on P19 measured by ABR. *B* and *C*, ABR waveforms of littermate WT mice (WT, *B*) and *Ednrb*^{−/−} (*Ednrb*^{−/−}, *C*) on P19 at 10–90 dB SPL of 12 kHz sound. *D–G*, immunohistochemical analysis of Ednrb expression in the cochlea (*D* and *E*) and the SV (*F* and *G*) from *Ednrb*^{−/−} mice (*E* and *G*) and littermate WT mice (*D* and *F*) on P19. *Arrows* and *arrowheads* in *D* and *E* indicate SGNs and the SV, respectively. *H*, percentage (means ± S.E.) of Ednrb expression in the SV from *Ednrb*^{−/−} mice (*Ednrb*^{−/−}, *blue bar*, *n* = 3) and littermate WT mice (WT, *white bar*, *n* = 3) to that in the SV from WT mice. *I* and *J*, LacZ staining of melanocytes in the SV. We employed *Dct-LacZ* mice, in which the *Dct* promoter is known as a specific marker of melanocytes (intermediate cells) (31), to establish *Dct-LacZ;Ednrb*^{−/−} mice newly by crossing *Ednrb*^{−/−} mice and *Dct-LacZ* mice (32). *K*, percentage (means ± S.E.) of LacZ-positive melanocytes in the SV from *Ednrb*^{−/−} mice (*Ednrb*^{−/−}, *n* = 3) and littermate WT mice (WT, *white bar*, *n* = 3) to that in the SV from WT mice. LacZ staining showed no positive cells in the SV from *Dct-LacZ;Ednrb*^{−/−} mice (*arrowhead* in *J* and *K*), whereas *Dct-LacZ* mice with intact Ednrb showed LacZ-positive melanocytes in the SV (*blue signals* indicated by *arrowhead* in *I* and *K*). Significant difference (*, *p* < 0.05; **, *p* < 0.01) from the control was analyzed by the Mann-Whitney *U* test. *Scale bars*: 100 μm (*D* and *E*) and 50 μm (*F–J*).

SV (Fig. 1*F*) in WT mice but not in *Ednrb*^{−/−} mice (Fig. 1, *E*, *G*, and *H*). Correspondingly, there were no melanocytes in the SV from *Ednrb*^{−/−} mice (Fig. 1, *I–K*).

Neurodegeneration of SGNs in Ednrb^{−/−} *Mice*—Immunohistochemical analysis of SGNs on P19 showed expression of Ednrb protein in WT mice (Fig. 2*C*) but not in *Ednrb*^{−/−} mice (Fig. 2*D*), whereas it was undetectable in SGNs from WT mice on P3 (Fig. 2, *A*, *B*, and *E*). Ednrb protein was not detectable in
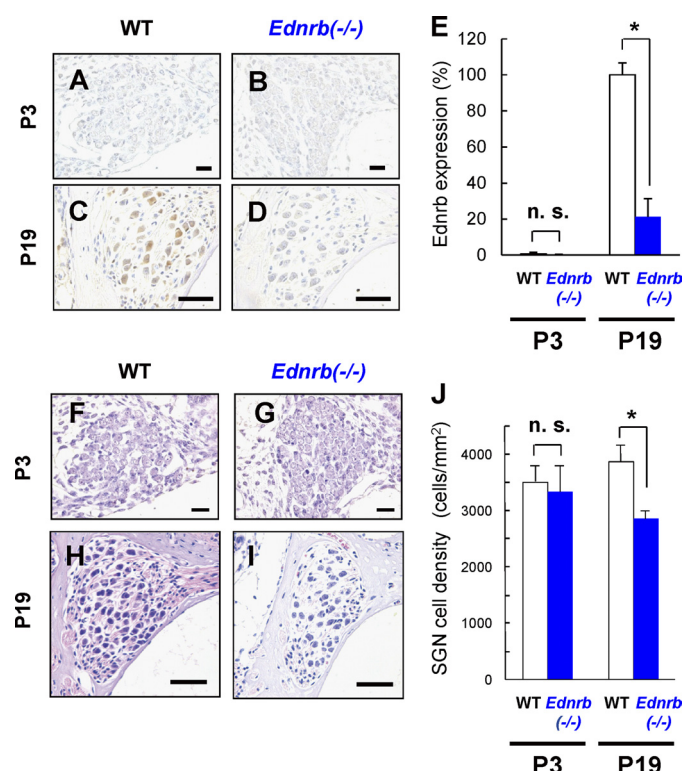

FIGURE 2. Decreased cell density of SGNs in *Ednrb*^{−/−} mice. *A–D*, immunohistochemical analyses for serial sections of the levels of Ednrb expression in SGNs from *Ednrb*^{−/−} mice (*B* and *D*) and littermate WT mice (*A* and *C*) on P3 (*A* and *B*) and P19 (*C* and *D*). *E*, percentage of positive-SGN number (means ± S.E. (*error bars*)) of Ednrb in *Ednrb*^{−/−} mice (*Ednrb*^{−/−}, *blue bar*, *n* = 3) and littermate WT mice (WT, *white bars*, *n* = 3) on P3 and P19 to that in WT mice on P19. *F–I*, H&E staining in SGNs at the basal turn from *Ednrb*^{−/−} mice (*G* and *I*) and littermate WT mice (*F* and *H*) on P3 (*F* and *G*) and P19 (*H* and *I*). Scale bars: 20 μm (*A*, *B*, *F*, and *G*) and 50 μm (*C*, *D*, *H*, and *I*). *J*, cell density (means ± S.E.) of SGNs from littermate WT mice (*white bars*) and *Ednrb*^{−/−} mice (*blue bars*) on P3 and P19. Significant difference (*, *p* < 0.05) from the control was analyzed by the Mann-Whitney *U* test. *n.s.*, not significant.

hair cells from WT mice (supplemental Fig. S1). Moreover, cell density of SGNs in the basal turn from *Ednrb*^{−/−} mice was 20–30% lower than that in the basal turn from littermate WT mice on P19 (Fig. 2, *H–J*) but not on P3 (Fig. 2, *F*, *G*, and *J*). Hair cells in the inner ear showed comparable morphology in WT and *Ednrb*^{−/−} mice on P19 (supplemental Fig. S2). We further performed detailed morphological analyses of SGNs from *Ednrb*^{−/−} mice and littermate WT mice on P16 by TEM analysis (Fig. 3). Gap areas (*arrows* in Fig. 3*B*) between SGNs (SGN in Fig. 3*B*) and Schwann cells were observed in *Ednrb*^{−/−} mice but not in littermate WT mice on P16 (*arrow* in Fig. 3*A*). SGNs from *Ednrb*^{−/−} mice showed vacuolar degeneration of the Golgi apparatus (*arrows* in Fig. 3*D*) and mitochondria (*arrowhead* in Fig. 3*D*), whereas WT mice showed intact morphology of the Golgi apparatus (*arrow* in Fig. 3*C*) and mitochondria (*arrowhead* in Fig. 3*C*). Because gaps between SGNs and Schwann cells and vacuolar degeneration have been shown to be neurodegeneration markers (13, 25), our results suggest that decreased cell density of SGNs in *Ednrb*^{−/−} mice was caused by neurodegeneration.

Improved Hearing Levels of Ednrb^{−/−};*DBH-Ednrb Mice*— Our results showed not only a defect in melanocytes of the SV (Fig. 1) but also neurodegeneration of SGNs (Fig. 2) in *Ednrb*^{−/−}
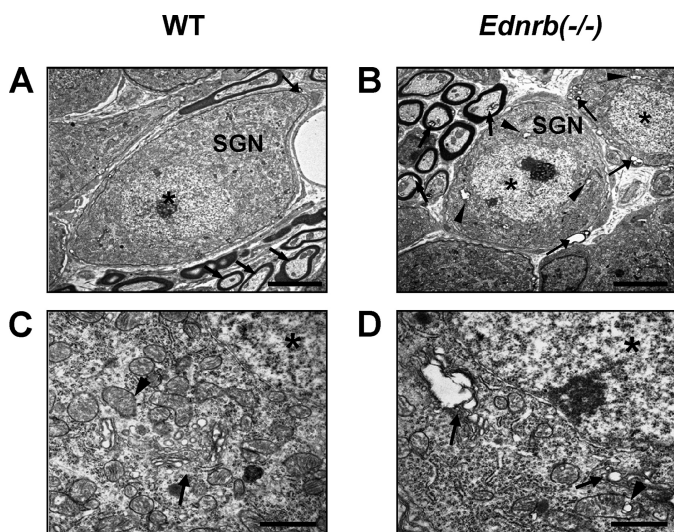
WT   *Ednrb(-/-)*

FIGURE 3. **Neurodegeneration of SGNs in *Ednrb*$^{-/-}$ mice.** TEM of SGNs from *Ednrb*$^{-/-}$ mice (*B* and *D*) and littermate WT mice (*A* and *C*) on P16. *A* and *B*, gap areas (*arrows* in *B*) between SGNs (SGN in *B*) and Schwann cells were observed in *Ednrb*$^{-/-}$ mice but not in littermate WT mice (*arrow* in *A*). *C* and *D*, vacuolar degeneration of the Golgi apparatus (*arrows* in *D*) and mitochondria (*arrowhead* in *D*) in SGNs from *Ednrb*$^{-/-}$ mice and intact morphology of the Golgi apparatus (*arrow* in *C*) and mitochondria (*arrowhead* in *C*) from WT mice. *Asterisks* indicate nuclei. *Scale bars*: 5 μm (*A* and *B*) and 1 μm (*C* and *D*).
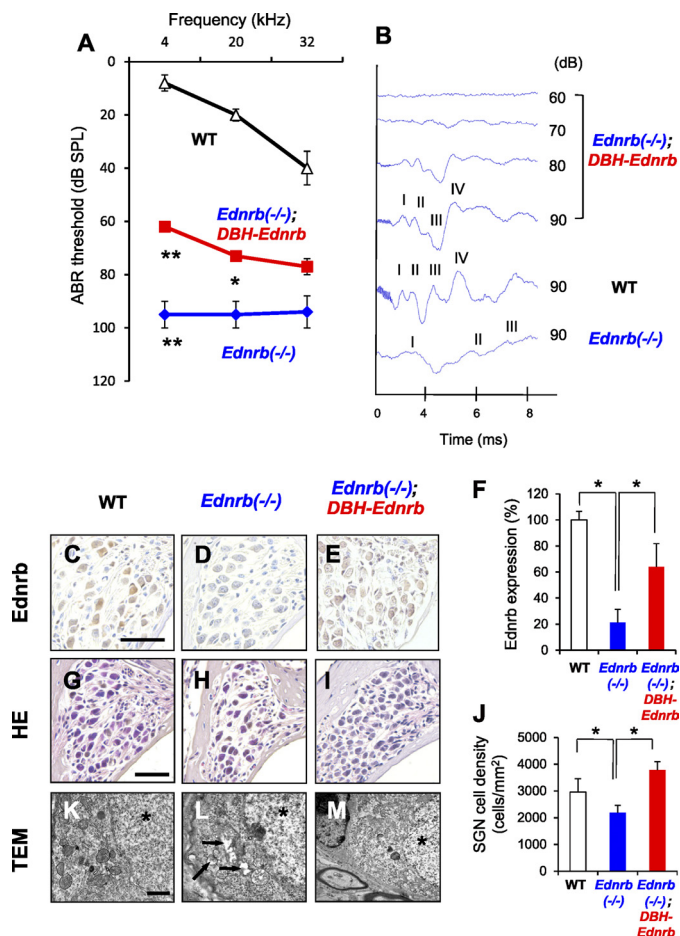

FIGURE 4. **Improvements of hearing levels in *Ednrb*$^{-/-}$ mice by *DBH-Ednrb* transgene.** *A*, hearing levels (means $\pm$ S.E. (*error bars*)) in WT ($n = 9$), *Ednrb*$^{-/-}$ ($n = 9$), and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice ($n = 12$) on P19 measured by ABR. *B*, ABR waveforms of littermate WT, *Ednrb*$^{-/-}$, and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice on P19 at 12-kHz sound. ABR wave peaks correspond to cochlear nerve activity (*wave I*) and downstream neural activities (*waves II–IV*) (33, 34). *C–E*, immunohistochemical analysis of Ednrb expression in SGNs from WT (*C*), *Ednrb*$^{-/-}$ (*D*), and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*E*) on P19. *F*, percentage of positive SGN number (means $\pm$ S.E.) of Ednrb in *Ednrb*$^{-/-}$ mice (*Ednrb*$^{-/-}$, *blue bar*, $n = 3$), *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*Ednrb*$^{-/-}$;*DBH-Ednrb*, *red bar*, $n = 3$) and littermate WT mice (WT, *white bar*, $n = 3$) to that in WT mice. *G–I*, H&E staining of SGNs at the basal turn from WT (*G*), *Ednrb*$^{-/-}$ (*H*), and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*I*) on P19. *J*, cell density (means $\pm$ S.E.) of SGNs from WT, *Ednrb*$^{-/-}$, and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice on P19. *K–M*, TEM of SGNs from WT (*K*), *Ednrb*$^{-/-}$ (*L*), and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*M*) on P16. Vacuolar degeneration in SGNs from *Ednrb*$^{-/-}$ mice (*arrows* in *L*) was not observed in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*M*). *Asterisks* indicate nuclei (*K–M*). *Scale bars*: 50 μm (*C–E*, *G–I*), 1 μm (*K–M*). Significant difference (*, $p < 0.05$; **, $p < 0.01$) from the control was analyzed by the Mann-Whitney *U* test.

mice. Previous studies showed that megacolon disease in *Ednrb*$^{-/-}$ mice was recovered in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice, suggesting that *Ednrb* transgene driven by the human *DBH* promoter recovers development of enteric ganglion neurons (18, 26). To clarify the role of Ednrb expressed in SGNs in neurodegeneration of SGNs, we next crossed *Ednrb*$^{-/-}$ mice with *DBH-Ednrb* transgenic mice (18, 26) to develop *Ednrb*$^{-/-}$; *DBH-Ednrb* mice. Comparative analysis of ABR thresholds between *Ednrb*$^{-/-}$ mice and littermate *Ednrb*$^{-/-}$;*DBH-Ednrb* mice on P19 revealed marked improvement (more than 30 dB SPL at 4 kHz) of hearing levels in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (Fig. 4*A*). Latencies of all four ABR waves in *Ednrb*$^{-/-}$ mice were also recovered in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (Fig. 4*B*). We next determined the rescue effect of *Ednrb* transgene driven by the *DBH* promoter on SGNs in *Ednrb*$^{-/-}$ mice. Comparable expression levels of Ednrb in SGNs were observed in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice and littermate WT mice on P19 (Fig. 4, *C–F*). Cell density of SGNs from *Ednrb*$^{-/-}$;*DBH-Ednrb* mice was also comparable with that of SGNs from littermate WT mice and was significantly higher than that of SGNs from *Ednrb*$^{-/-}$ mice (Fig. 4, *G–J*). Gaps between SGNs and Schwann cells and vacuolar degeneration of the Golgi apparatus and mitochondria were nearly undetectable by TEM in *Ednrb*$^{-/-}$; *DBH-Ednrb* mice as well as WT mice (Fig. 4, *K–M*). These results suggest that not only enteric ganglion neurons but also SGNs in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice were recovered by *Ednrb* transgene driven by the *DBH* promoter.

Defects of Melanocytes in the SV from Ednrb$^{-/-}$*;DBH-Ednrb Mice*—We finally examined whether *Ednrb* transgene driven by the *DBH* promoter affects defects of melanocytes in the SV from *Ednrb*$^{-/-}$ mice. Neither Ednrb-positive cells (Fig. 5, *A–C*, *G*) nor Kir4.1-positive cells (Fig. 5, *D–F*, *H*), a specific marker of melanocytes (27), were detectable in the SV from *Ednrb*$^{-/-}$ mice (Fig. 5, *B*, *E*, *G*, and *H*) and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice

(Fig. 5, *C*, *F*, *G*, and *H*). TEM analysis further revealed that *Ednrb*$^{-/-}$ mice and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice similarly exhibited no melanocytes and many gap areas (*red arrows* in Fig. 5, *J* and *K*) among marginal cells and blood vessels, whereas WT mice showed melanocytes among marginal cells and blood vessels (Fig. 5*I*). In addition, EP of *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (79 $\pm$ 6 mV) was significantly ($p < 0.01$) lower than that of WT mice (109 $\pm$ 4 mV) (supplemental Fig. S4). These results suggest that defects of melanocytes in the SV from *Ednrb*$^{-/-}$ mice were not recovered by *Ednrb* transgene driven by the *DBH* promoter. Correspondingly, there was no difference in coat color or defects of melanocytes between *Ednrb*$^{-/-}$ mice and *Ednrb*$^{-/-}$; *DBH-Ednrb* mice (supplemental Fig. S5). These results suggest
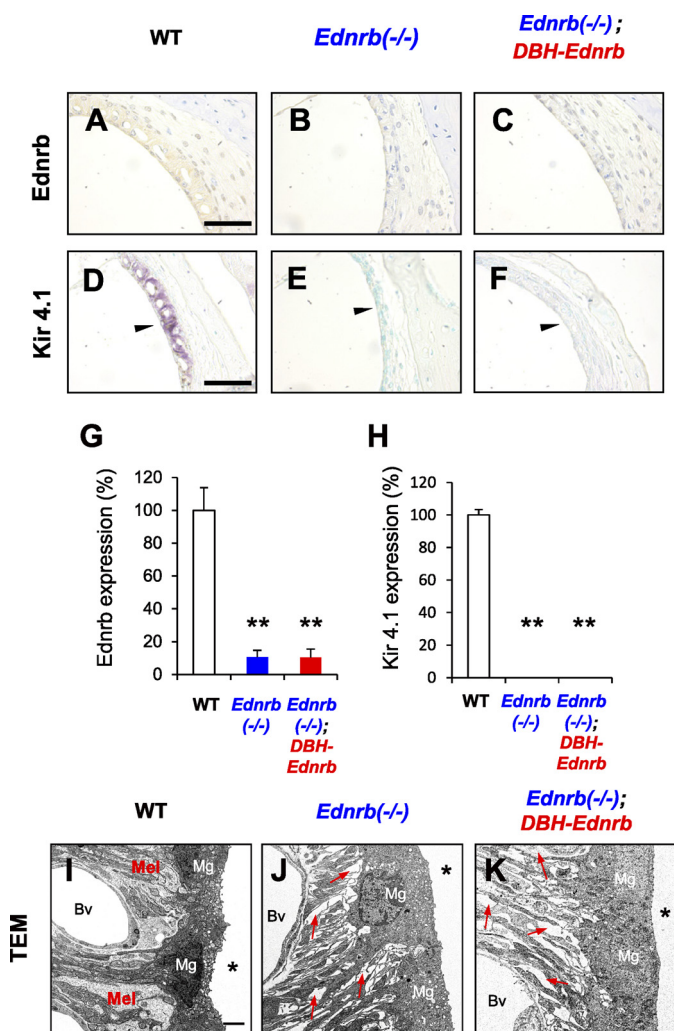
FIGURE 5. **Melanocyte defect in the SV from *Ednrb*$^{-/-}$ mice was not rescued in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice.** *A–C*, immunohistochemical analysis of Ednrb expression in the SV from WT mice (*A*), *Ednrb*$^{-/-}$ mice (*B*), and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*C*) on P19. *D–F*, immunohistochemical analysis of Kir4.1 expression, which is known as one of the melanocyte markers in the SV (27). Kir4.1-expressing cells were found in WT mice (*purple signals* indicated by *arrowhead* in *D*) but were not found in *Ednrb*$^{-/-}$ mice and *Ednrb*$^{-/-}$;*DBH-Ednrb mice* (*E* and *F*). The methods used for staining are described in detail under "Experimental Procedures." *G* and *H*, percentage (means ± S.E. (*error bars*)) of Ednrb (*G*) and Kir4.1 (*H*) expression levels in the SV from *Ednrb*$^{-/-}$ mice (*Ednrb*$^{-/-}$, *blue bar*, *n* = 3), *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*Ednrb*$^{-/-}$;*DBH-Ednrb*, *red bar*, *n* = 3) and littermate WT mice (WT, *white bar*, *n* = 3) to that in the SV from WT mice. Significant difference (*, *p* < 0.05; **, *p* < 0.01) from the control was analyzed by the Mann-Whitney *U* test. *I–K*, TEM of the SV from *Ednrb*$^{-/-}$ mice (*J*), *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (*K*), and littermate WT mice (*I*) on P19. WT mice exhibited melanocytes (Mel in *I*) among marginal cells (*Mg* in *I*) and blood vessels (*Bv* in *I*), whereas *Ednrb*$^{-/-}$ mice and *Ednrb*$^{-/-}$;*DBH-Ednrb* mice exhibited no melanocytes and many gaps (indicated by *red arrows* in *J* and *K*) among marginal cells (*Mg* in *J* and *K*) and blood vessels (*Bv* in *J* and *K*). *Asterisk* indicates endolymphatic space (*I–K*). *Scale bars*: 50 μm (*A–F*), 2 μm (*I–K*).

that Ednrb transgene driven by the *DBH* promoter did not affect development of melanocytes in the skin and SV.

DISCUSSION

This study demonstrated that *Ednrb*$^{-/-}$ mice had severe congenital deafness (ABR threshold > 90 dB SPL) with not only a defect of melanocytes in the SV (Figs. 1 and 5) but also neurodegeneration of SGNs (Figs. 2 and 3). These results indicate a novel etiology for Ednrb-mediated hearing loss in *Ednrb*$^{-/-}$ mice that involves degeneration of SGNs, which serve as peripheral neurons in inner ears, besides defects of melanocytes in the SV.

This study showed neurodegeneration of SGNs resulting in decreased numbers of SGNs in *Ednrb*$^{-/-}$ mice on P19 (Figs. 2 and 3), whereas cell density and morphology of SGNs were comparable in *Ednrb*$^{-/-}$ mice and WT mice on P3 (Fig. 2). These results suggest that SGNs in *Ednrb*$^{-/-}$ mice developed normally at least until P3, when the level of Ednrb expression in SGNs from WT mice was undetectable (Fig. 2*A*). However, SGNs from *Ednrb*$^{-/-}$ mice no longer survived on P19 (Fig. 2, *I* and *J*), when the level of Ednrb expression in SGNs from WT mice was clearly detectable (Fig. 2*C*). We therefore conclude that a defect of Ednrb expression affects survival of SGNs during hearing development after birth in mice.

Degeneration of SGNs but not the defect of melanocytes in the SV from *Ednrb*$^{-/-}$ mice was recovered in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice (Figs. 4 and 5). The defect of Ednrb protein in SGNs, but not in melanocytes in the SV (Fig. 5) or in inner and outer hair cells (supplemental Fig. S1), from *Ednrb*$^{-/-}$ mice was correspondingly rescued by *Ednrb* transgene driven by the *DBH* promoter. In addition, the suprathreshold ABR, which has been shown to reflect auditory nerve activity (28), showed similar growth rates in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice and littermate WT mice (supplemental Fig. S6), suggesting that development of SGNs was similar in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice and WT mice. On the other hand, *Ednrb*$^{-/-}$;*DBH-Ednrb mice* showed a significantly lower level of EP than that in WT mice (supplemental Fig. S4), although a previous study has shown that there is a link between EP levels and auditory nerve activities (29). TEM analysis also showed no melanocytes with many gap areas in the SV from *Ednrb*$^{-/-}$;*DBH-Ednrb* mice as well as *Ednrb*$^{-/-}$ mice (Fig. 5, *I–K*), suggesting impairments of the SV in *Ednrb*$^{-/-}$; *DBH-Ednrb* mice. Thus, these results suggest that degeneration of SGNs in *Ednrb*$^{-/-}$ mice was specifically recovered in inner ears of *Ednrb*$^{-/-}$;*DBH-Ednrb* mice. We further showed that hearing levels in *Ednrb*$^{-/-}$;*DBH-Ednrb* mice were partially (20 –30 dB SPL) recovered compared with those in *Ednrb*$^{-/-}$ mice (Fig. 4). Thus, these results suggest that Ednrb expressed in SGNs is partially required for postnatal development of hearing.

Ednrb has been reported to mediate embryonic development of melanocytes (30) and the enteric nervous system (18, 26) derived from the neural crest. Our results indicate a novel possibility that Ednrb is essential for postnatal development of SGNs, although the development process of SGNs (*e.g.* differentiation or migration of precursors) during prenatal and postnatal hearing development has not been completely elucidated (12). Our previous study also showed that impairment of c-Ret causes severe congenital hearing loss with degeneration of SGNs and with intact morphology of hair cells and the SV (13). Because both *EDNRB* and c-*RET* cause megacolon disease with congenital intestinal aganglionosis in mice and humans, further study is needed to determine whether megacolon-related molecules such as *SOX10* and *PAX3* are involved in congenital hearing loss caused by degeneration of SGNs.

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The degeneration of SGNs from *Ednrb*$^{-/-}$ mice did not involve the hallmark of apoptotic signals (supplemental Fig. S3). The results of a previous study also showed that neurodegeneration of enteric neurons did not involve apoptotic signals during the developmental stage in mice with deletion of *Ednrb* (30). On the other hand, our results showed that hair bundles of inner and outer hair cells in *Ednrb*$^{-/-}$ mice, which have already developed congenital hearing loss, were comparable with those in littermate WT mice (supplemental Fig. S2). Immunohistochemical analysis correspondingly showed that expression of Ednrb protein was nearly undetectable in hair cells from WT mice (supplemental Fig. S1). These results suggest that the congenital hearing loss in *Ednrb*$^{-/-}$ mice involves postnatal degeneration of SGNs as well as defects of melanocytes in the SV rather than disturbance of hair cells.

Several mouse models for *Ednrb*-mediated WS-IV have been reported (summarized in supplemental Fig. S7). *sl* mice, in which exon 1 and intron 1 are spontaneously deleted, and WS-IV mice, in which exons 2 and 3 are spontaneously deleted, have been shown to develop megacolon disease and hearing loss. On the other hand, the hearing level of *Ednrb*$^{-/-}$ mice with deletion of exon 3, which we analyzed in this study, has not been reported. In humans, although impairments of *EDNRB* caused by nonsense mutations of exon 3 have been reported also to result in the development of WS with hearing loss, the etiology has not been clarified. Thus, this study for the first time provides an insight into the pathogenesis of congenital hearing loss caused by impairment of *Ednrb*$^{-/-}$ by deletion of exon 3 in mice.

Our results suggest that 60–70 dB SPL of hearing levels could be maintained even if there are no melanocytes in the SV in inner ears of *Ednrb*$^{-/-}$;DBH-Ednrb mice. Because a previous study has shown that a transgene driven by the *Dct* promoter is expressed in melanocytes (31), further study is needed to determine the concurrent rescue effect of *Ednrb* transgene driven by the *Dct* promoter and the *DBH* promoter on congenital deafness in *Ednrb*$^{-/-}$ mice.

In summary, this study demonstrates a novel role of Ednrb expression in SGNs distinct from that in melanocytes in the SV contributing partially to postnatal hearing development via survival of SGNs. A therapeutic strategy for congenital hearing loss in WS-IV patients has not been established. Enhancement of *EDNRB* expression in SGNs could be a novel potential therapeutic strategy for congenital hearing loss in WS-IV patients.

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