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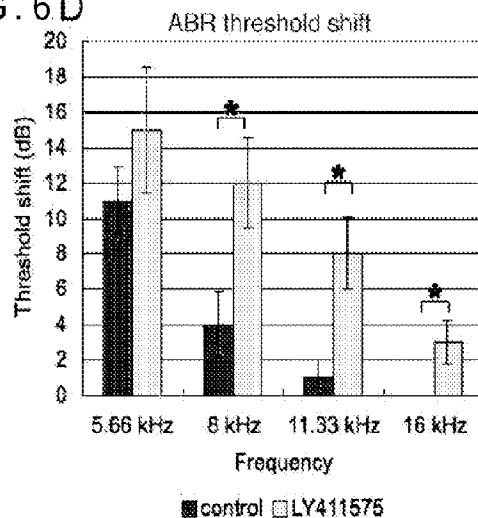
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(54) Title: TREATING HEARING LOSS

FIG. 6D



(57) Abstract: This invention relates to methods for treating hearing loss associated with loss of cochlear hair cells, e.g., caused by noise exposure, using certain gamma secretase inhibitors, in post-neonatal animals, e.g., adolescents and adults.

## Treating Hearing Loss

### CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 61/698,475, filed on September 7, 2012. The entire contents of the foregoing are  
5 incorporated herein by reference.

### FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant Nos. RO1 DC007174, R21 DC010440-01 and P30 DC05209 awarded by the National Institutes of Health. The Government has certain rights in the invention.

### TECHNICAL FIELD

This invention relates to methods for treating hearing loss associated with loss of cochlear hair cells, e.g., caused by noise exposure, in post neonatal animals, e.g., adolescent or adult animals, using certain Notch inhibitors, e.g., gamma secretase inhibitors.

### BACKGROUND

The cochlear sensory epithelium contains hair cells adapted for the detection of sound, which is transduced by stereocilia at their apical surfaces (1, 2). Hair cells produced during development are post-mitotic and are not replaced after loss (3-6) or as part of normal cell turnover in mammals (7-9). As a result, deafness due to hair  
20 cell loss is irreversible. Hair cell development during the embryonic period includes a complex series of fate decisions, in which prosensory epithelial cells acquire different fates, either hair cell or supporting cell, through a process of lateral inhibition which is mediated by Notch signaling (5, 10, 11). Supporting cells are prevented from  
25 differentiating into hair cells by active Notch signaling stimulated by ligands on adjacent hair cells. This active Notch signaling ends shortly after birth, given the loss of an effect of  $\gamma$ -secretase inhibitors on hair cell number in the early postnatal period (13) and other data suggesting that Notch signaling is extinguished after birth (14).

## SUMMARY

Hearing loss due to damage to auditory hair cells is normally irreversible because mammalian hair cells do not regenerate after the newborn period. At least in part, the present invention is based on the discovery that blocking Notch signaling with certain gamma-secretase inhibitors resulted in regeneration of cochlear hair cells in adult animals that correlated with recovery of hearing after noise-induced hearing loss.

Thus, in one aspect the invention features methods for treating hearing loss caused by loss of cochlear hair cells in a post-neonatal mammal. The methods include systemically or locally administering to the ear of the mammal a composition comprising a therapeutically effective amount of a Notch inhibitor, e.g., a gamma secretase inhibitor, wherein the therapeutically effective amount is an amount sufficient to restore hearing at one or more frequencies.

In some embodiments, the hearing loss was caused by exposure to a physical or chemical ototoxic insult, e.g., repeated (chronic) exposure or one or more acute exposures.

In some embodiments, the physical ototoxic insult is noise.

In some embodiments, the composition is administered to the ear within four weeks, two weeks, one week, or one day of the exposure to the insult.

In some embodiments, the composition is applied topically to the round window.

In some embodiments, the composition further comprises a carrier, e.g., a sustained release carrier. In some embodiments, the carrier is a polyoxyethylene-polyoxypropylene triblock copolymer.

In some embodiments, the composition comprises at least 10 mM of the Notch inhibitor.

In some embodiments, the methods further include determining a baseline level of hearing at one or more frequencies before administering the composition, and a subsequent level of hearing at the same one or more frequencies after administering the composition, and administering one or more additional doses of the composition until a desired level of hearing at the one or more frequencies is recovered. In some embodiments, the subsequent level of hearing is determined one week, two weeks,

three weeks, one month, two months, three months, four months, six months, and/or twelve months after administering the composition.

In some embodiments, the gamma secretase inhibitor is selected from the group consisting of RO4929097; DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester); L-685458 ((5S)-(t-Butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl)-L-leu-L-phe-amide); BMS-708163 (Avagacestat); BMS-299897 (2-[(1R)-1-[[[4-Chlorophenyl)sulfonyl](2,5-difluorophenyl)amino]ethyl-5-fluorobenzenebutanoic acid); MK-0752; YO-01027; MDL28170 (Sigma); LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide, see US 6,541,466); ELN-46719 (2-hydroxy-valeric acid amide analog of LY411575 (where LY411575 is the 3,5-difluoro-mandelic acid amide) (US Patent No 6,541,466)); PF-03084014 ((S)-2-((S)-5,7-difluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-N-(1-(2-methyl-1-(neopentylamino)propan-2-yl)-1H-imidazol-4-yl)pentanamide, Samon et al., Mol Cancer Ther 2012;11:1565-1575); Compound E ( (2S)-2-[[[(3,5-Difluorophenyl)acetyl]amino}-N-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl]propanamide; see WO 98/28268 and Samon et al., Mol Cancer Ther 2012;11:1565-1575); and Semagacestat (LY450139; (2S)-2-hydroxy-3-methyl-N-((1S)-1-methyl-2-[[[(1S)-3-methyl-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-1-yl]amino}-2-oxoethyl)butanamide), or pharmaceutically acceptable salts thereof.

In some embodiments, the gamma secretase inhibitor is LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide).

In some embodiments, the post-neonatal mammal is a child, adolescent or adult, e.g., above the age of 6 months, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 years.

In some embodiments, the mammal is an adult of at least 40 years of age, e.g., at least 45, 50, 55, 60, 65, 70 years of age.

In some embodiments, the mammal is a human.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also

be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will  
5 control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

### DESCRIPTION OF DRAWINGS

The patent or application file contains at least one drawing executed in color.  
10 Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

#### **Figures 1A-D. *In vitro* activity of $\gamma$ -secretase inhibitors in hair cell induction**

(A) Relative ratio of nGFP-positive cells to DAPI-positive cells after treatment  
15 of inner ear spheres made from *Math1-nGFP* mice with  $\gamma$ -secretase inhibitors at the indicated concentrations ( $\mu$ M) reveals that LY411575 had the greatest potency of 4 inhibitors tested for hair cell induction. Data were normalized to control values obtained by addition of DMSO. Asterisks indicate  $p < 0.01$ .

(B) Ratio of myosin VIIa (labels hair cells) to Hoechst-positive cells induced  
20 by LY411575 was calculated relative to DMSO-treated spheres from organ of Corti.

(C) Explant cultures of the organ of Corti from P1 mice cultured for 72 h in the presence of DMSO or LY411575 (1  $\mu$ M) had ectopic hair cells (myosin VIIa; green) in the outer hair cell region (white bracket). Ectopic hair cells were positive for phalloidin (labels the hair bundle and cuticular plate; shown in red). Inset is a  
25 high-power view (scale bar is 2  $\mu$ M) of a phalloidin-stained hair cell showing bundle structure.

(D) An increase in myosin VIIa-positive cells per 100  $\mu$ m of the cultured organ of Corti explants from P1 mice was found 72 h after LY411575 treatment.

In all graphs, error bars show the standard error of the mean. Scale bar is 50  
30  $\mu$ m.

**Figures 2A-D. Hair cell replacement after LY411575 treatment of organ of Corti explants from mice subjected to ablation of hair cells**

(A) Hair cells can be seen throughout the neonatal organ of Corti in a whole mount labeled for myosin VIIa.

5 (B) Three rows of outer (white bracket; OHC1 - 3) and one row of inner hair cells (IHC) can be seen in a P3 organ of Corti explant after staining for myosin VIIa. Deiters' cells (DC1 - 3) and Hensen cells (HC) in the outer hair cell region are positive for Sox2.

10 (C) Organ of Corti explants from *Pou4f3-Cre; Mos-iCsp3* double-transgenic mice subjected to dimerizer-induced hair cell ablation and cultured for 3 d in the presence of LY411575 had an increased number of myosin VIIa-positive cells in the outer hair cell region (white bracket) compared to the carrier-treated explant. The same region had a decreased number of Sox2-positive cells relative to the control. A high power view (scale bar, 2  $\mu$ M) of phalloidin-stained tissue shows the hair cell stereociliary bundles (inset).

15 (D) The number of outer hair cells at the mid-apex and mid-base was increased in the LY411575 treated as compared to the control cochleae in the hair cell-ablated samples (Csp Tg). Increased numbers of hair cells were also seen after LY411575 treatment of wild-type organ of Corti (Wt) at the apex, mid-apex and mid-  
20 base. In both cases the increase in the number of hair cells was accompanied by a decrease in the number of supporting cells.

The error bars are standard error of the mean (n = 7 in each group). Asterisks indicate  $p < 0.05$ . All scale bars are 50  $\mu$ m.

25 **Figures 3A-B. Time course of *Hes5* and *Atoh1* mRNA expression in the cochlea with or without LY411575 after noise exposure**

(A) Elevated levels of *Hes5* after noise exposure were diminished in response to LY411575 treatment and reached the pre noise level. Without inhibitor, expression levels of *Hes5* in the cochlea increased 1 d after noise exposure and remained elevated  
30 compared to the pre noise level for up to 2 d. Samples for qRT-PCR were taken before exposure to noise (pre noise), at the time (day 0) of drug treatment (post noise), at day 1 of drug treatment (d 1), day 3 of drug treatment (d 3), and day 7 of drug

treatment (d 7). mRNA expression levels were calculated relative to the pre-noise level.

(B) Treatment with LY411575 significantly increased the expression of *Atoh1* compared to the opposite, untreated ear 1 d after noise exposure. Increased levels were detected 1 d after drug treatment (d 1) and remained elevated 3 d after drug treatment (d 3; n = 9 in each group).

Error bars are standard error of the mean. Asterisks indicate  $p < 0.05$ .

**Figures 4A-G. Lineage tracing of supporting cells in noise-exposed cochleae treated *in vivo* with a  $\gamma$ -secretase inhibitor**

(A) Double-labeled cells (arrowheads) positive for Sox2 lineage (GFP) and myosin VIIa (blue) were observed in the outer hair cell area (white bracket) in cochlear tissues from deafened mice carrying the *Sox2-CreER* as well as the *Cre* reporter transgene, *mT/mG*, 1 month after LY411575 treatment. Hair cell co-labeling with the lineage tag indicates derivation from a Sox2-positive cell and is thus evidence for regenerated hair cells after deafening in the mature mouse cochlea by transdifferentiation of supporting cells. These confocal xy-projection images of LY411575-treated ears from *Sox2-CreER*; *mT/mG* double transgenic mice are in the 8 kHz area of the cochlear longitudinal frequency map.

(B) Confocal xz-projections from the same area as A show that myosin VIIa-positive cells in the medial part of the outer hair cell area (white bracket) had GFP-positive hair bundle structures, indicating a Sox2 lineage (arrowhead). The cell shown was attached to the basement membrane (arrow) similar to a supporting cell.

(C) Cells double-labeled for myosin VIIa (blue) and Sox2 lineage (green) were observed (arrowheads) in the outer hair cell area (white bracket) in the 11.3 kHz region in this xy projection from a deafened cochlea 1 month after LY411575 treatment. Original hair cells have red hair bundles and new, Sox2-lineage hair cells have green (GFP-positive) bundles. The higher power view (inset, scale bar is 2  $\mu$ M) shows hair cells with their original

(red) bundles adjacent to cells derived from Sox2-positive cells (green bundles).

(D) Cross section from the same area as C show that myosin VIIa-positive cells in the outer hair cell area (white bracket) had GFP-positive hair bundle

structures. The cell shown is attached to the basement membrane (arrow). Note position of the nucleus close to the basement membrane.

(E) No double-labeled cells were found in the xy-projection images from the control (contralateral to A) ear in the 8 kHz area. White bracket shows the outer hair cell region.

(F) xz-projections confirm the lack of double-labeled hair cells in the control, contralateral ear.

All scale bars are 50  $\mu\text{m}$ .

(G) Quantification of the GFP (Sox2 lineage) and myosin VIIa double-labeled cells in the outer hair cell region 1 month after treatment with LY411575 in deafened mice at frequency-specific cochlear areas ( $n = 5$  in each group). Error bars are standard error of mean.

#### **Figures 5A-B. Hair cells in damaged mature cochlea treated with LY411575 *in vivo***

(A) The number of hair cells (green; myosin VIIa) in the outer hair cell region (white brackets) of deafened cochleae at 8, 11.3, and 16 kHz areas was increased compared to the control ear (right ear treated with carrier) 3 months after treatment with LY411575 (left ear), and the increase was accompanied by a decrease in the number of supporting cells (blue: Sox2) in the same regions in these whole mount confocal xy-projections.

(B) Significant differences in the numbers of hair cells and supporting cells were observed in the outer hair cell area at 8 and 11.3 kHz regions of treated (left) ears 3 months after treatment with LY411575 as compared to the values in the contralateral carrier-treated ear of deafened mice ( $n = 5$  in each group).

All scale bars are 50  $\mu\text{m}$ . Error bars are standard error of the mean and asterisks indicate  $p < 0.05$ .

#### **Figures 6A-E. Measurement of ABR in deafened ears after LY411575 treatment**

(A, B) A decrease in ABR thresholds at low frequencies (up to 16 kHz) in the left, LY411575-treated ear (B) compared to the right, control ear (A) was apparent in ABR thresholds in recordings made at 7 frequencies from 5.66 to 45.25 kHz with the

following time course. Before noise exposure (Pre Noise: *open circles*), 1 d after noise exposure (Post Noise: *filled circles*), 1 week after drug treatment (1 W: *open squares*), 1 month after treatment (1 Mo: *crosses*), and 3 months after treatment (3 Mo: *filled triangles*) (n = 5 in each group). When no response was observed at 80 dB (maximum acoustic output of the system) the threshold was designated as 85 dB.

(C) An example of 8 kHz ABR waves recorded 3 months after drug treatment from the same mouse. Arrowheads show the peaks with the largest peak-to-peak amplitude. In the LY411575-treated ear, the peak could first be detected at 65 dB, while on the control side the peak could first be detected at 75 dB.

(D, E) The differences in threshold (D) and wave I amplitude (E) 3 months after drug treatment compared to 1 d after noise exposure between control and LY411575-treated ears at 8, 11.33, and 16 kHz (asterisks) were significant (n = 5 in each group).

Error bars are standard error of the mean.

#### **Figures 7A-C. Cochlear architecture in a mouse exposed to 8–16 kHz octave-band noise at 116 dB SPL for 2 h**

(A) The loss of hair cells (myosin VIIa) was apparent throughout the cochlea with the greatest loss seen in outer hair cells as well as a loss of inner hair cells in the 22 kHz region.

(B) At this noise exposure intensity loss of hair cells was accompanied by preservation of supporting cells (Sox2) in most of the cochlea, with the exception of the 22 kHz region, where inner hair cell damage was also seen.

(C) Merged images show the base (Piece 1), mid-base (Piece 2), mid-apex (Piece 3), and apex (Piece 4) and the corresponding frequencies.

Scale bar is 500  $\mu\text{m}$ .

#### **Figures 8A-B. Analysis of the effect *in vivo* on the brainstem response and hair cell morphology of LY411575 administered systemically to young adult noise-damaged mice**

(A) Treatment with LY411575 by injection intraperitoneally at 50 mg/kg daily for 5 d increased the responsiveness of the ear at low frequency after 2-4 months.

Significant improvements in threshold of the ABR were found between 1 and 3 months at 4, 8 and 16 kHz.

(B) Stereociliary bundles could be detected on hair cells after phalloidin staining (white arrows). Cells stained for myosin VIIa were increased in the apex of the cochlea where outer hair cell loss was incomplete.

Asterisks indicate  $p < 0.05$ . Scale bars are 50  $\mu\text{m}$ .

**Figure 9. Labeling of a mature mouse cochlea with the reporter strain**

The *Sox2-CreER* mouse crossed to the reporter line, *mT/mG*, treated with tamoxifen at P1 was examined as a whole mount after dissection. Any cells that express Sox2 at P1 would be expected to be GFP-positive after removal of the STOP sequence, whereas cells that do not will retain the tomato label. Supporting cells from the 5 to the 45 kHz regions are labeled by GFP from the Sox2-Cre reporter (Sox2-lineage; green). In contrast, myosin VIIa-labeled hair cells (blue) display td-Tomato labeling (td-Tomato; red), and this pattern is retained from the 5 to the 45 kHz region (see bundles in the merged image; Merge). Note that some pillar cells are not labeled by the GFP from the reporter (22.6 kHz for example), presumably due to incomplete Cre activity.

Scale bar is 50  $\mu\text{m}$ .

**Figure 10. Structures of Gamma-Secretase Inhibitors.**

The structures of DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester), L-685458 ((5S)-(t-Butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl)-L-leu-L-phe-amide), BMS-708163 (Avagacestat); MK-0752; YO-01027; MDL28170 (Sigma), LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide), and Semagacestat (LY450139; (2S)-2-hydroxy-3-methyl-N-((1S)-1-methyl-2-[(1S)-3-methyl-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-1-yl]amino)-2-oxoethyl)butanamide) are shown.

**DETAILED DESCRIPTION**

The generation of physiologically active hair cells in an adult mammal has been a sought-after but elusive goal. Transfection of bHLH transcription factor, *Atoh1*, which drives hair cell differentiation during development, is one approach that

increases hair cell number in embryonic or newborn tissue, but cells that were competent to become hair cells in the embryo lost their responsiveness as the animal matured (13, 17, 26). Delivery of Atoh1 in an adenovirus to the damaged, adult cochlea (16) showed some hair cell differentiation, but the number of new hair cells was not clear and new hair cells could not be traced from their precursors, making it difficult to distinguish between “new” hair cells and hair cells that had recovered from trauma due to a toxin or noise damage. Stimulation of cell division by silencing cell cycle inhibitors has been suggested as an alternative route to hair cell regeneration (6), but hair cells, due to their highly differentiated state, tend to activate suicide programs after they divide and proliferation can cause deafness (3, 27, 28). Regeneration of hair cells is made difficult by the cellular organization of the cochlea: minute changes in the interactions between cells of the epithelium are a cause of deafness (29). Tight junctions are required for maintaining the ionic milieu of endolymph that bathes the surface of hair cells, and the flexibility and spacing of outer hair cells has an impact on the function of the cochlear amplifier, which is achieved by outer hair cell contraction, and together with sound detection by the transduction apparatus of inner hair cells, accounts for the sensitivity and broad dynamic range of mammalian hearing (1, 30, 31).

The present inventors had recently shown that inhibition of Notch increased hair cell differentiation from stem cells and that the mechanism was dependent on Atoh1, since silencing the transcription factor in the  $\gamma$ -secretase inhibitor-treated stem cells prevented the induction of hair cell fate (15). As described herein, inner ear stem cells were used to select a potent  $\gamma$ -secretase inhibitor. The Notch pathway was targeted, as a strategy that would only be effective on cells that were actively signaling through Notch. Although increased Notch signaling in the adult after damage had been suggested by some (12), the loss of an effect of  $\gamma$ -secretase inhibitors on hair cell number in the early postnatal period (13) and data suggesting that Notch signaling was extinguished after birth (14) both suggested that  $\gamma$ -secretase inhibitors would have no effect on hair cell number in the adult mammalian cochlea. Thus, treating hearing loss by generating new hair cells would be difficult, particularly in light of the failures of previous attempts to make new hair cells by manipulating the cell cycle or by Notch gene knockouts in the embryo, which lead to the presence of large numbers of extra hair cells – but deaf animals, as the

supernumerary hair cells are not functional and can in fact damage the structures of the inner ear.

Surprisingly, as described herein, inhibition of Notch after noise damage leads to recovery of hearing ability; without wishing to be bound by theory, it is believed that this occurs by transdifferentiation of supporting cells into hair cells in post-neonatal animals. The basal location of the nucleus in the new hair cells was consistent with derivation from supporting cells, which are normally located in a plane below that of the hair cells. Supporting cell transdifferentiation was induced by Atoh1, which may be acting in a similar capacity to transcription factors, some of which are somewhat related to Atoh1, that allow cellular reprogramming and transdifferentiation to neurons (32, 33). The supporting cells express stem cell markers such as Sox2, Musashi1, and GLAST (34-36) and have the capacity for proliferation and transdifferentiation for a short period postnatally (26). Capacity for neurosphere formation by the sensory epithelial cells in the cochlea is found in a similar postnatal time frame (37).

Drug therapy for restoration of hair cells is a new approach and delivery to the inner ear fluids without actual injection into the cochlea may be an advantage over gene therapy and may also effectively restrict hair cell differentiation to cells in the sensory epithelial area as compared to gene therapy that may convert hair cells in a broader area. A middle ear approach was used for the delivery of LY411575 to the damaged inner ear; surprisingly, this route allowed delivery of a sufficient dose to have a therapeutic effect. Since the round window membrane consists of cell layers, lipid solubility of the drug favors permeability (23, 24).

Surprisingly, though previously it would have been predicted that the new cells would die, recovery lasted for at least 3 months, the longest time point measured.

Novel approaches using inner ear stem cells and transgenic mice were critical for the present demonstration that hair cells could regenerate in the mouse. The caspase-3 mouse provided a model in which hair cells could be selectively deleted without damage to other cells so that new hair cells could be accurately quantified. Lineage tracing with the *mT/mG*; *Sox2-CreER* double transgenic mouse allowed the unambiguous demonstration that drug treatment resulted in the generation of new hair cells and not recovery of hair cell bundles that could have accounted for recovery in the absence of lineage tracing. Improved thresholds were found by ABR, showing

that hearing was improved by  $\gamma$ -secretase inhibitor administration in the acute damage situation. Hair cell counts showed an increase in the same frequency regions as the improved ABR. Thus the frequency specificity of the improved hearing was used to determine the correlation between the gain in hair cell number and the improved hearing threshold. The damage in the acute noise exposure model reflected hair cell loss in humans, most severe in the base and restricted primarily to the outer hair cells (22). The improvement in threshold at the apex of the cochlea was thought to result from an increase in the number of hair cells to a level that produced a detectable change through outer hair cell activity. As a result of the greater damage at the base of the cochlea, the number of hair cells at the base was not adequate to lower the threshold of the ABR, and the increase in hair cells in the apex could not be detected by a change in DPOAE threshold. The combined physiological and cellular evidence allowed a definitive proof of the regeneration of hair cells that was quantitative, was correlated to frequency, and provided unequivocal evidence as to the genesis of the hair cells by lineage tracing from supporting cells.

#### *Methods of Treatment*

The compounds and methods described herein are appropriate for the treatment of post-neonatal (e.g., child, adolescent or adult, e.g., above the age of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 years) mammalian (e.g., human) subjects who have or are at risk of developing hearing disorders resulting from cochlear hair cell loss. The methods described herein can be used to treat cochlear hair cell loss and any disorder that arises as a consequence of hair cell loss in the ear, such as hearing impairments or deafness. These subjects can receive treatment with an agent described herein. The approach may be optimal for treatment of acute hearing loss shortly after the damage has occurred, and may be less effective after longer time periods when Notch signaling has returned to its baseline level in the adult.

In some embodiments, the methods include steps of selecting a patient at risk of cochlear hair cell loss and/or a patient with cochlear hair cell loss. Alternatively or in addition, the methods include steps of selecting a patient at risk of hair cell loss and/or a patient with cochlear hair cell loss. For example, any human experiencing or at risk for developing cochlear hair cell loss is a candidate for the treatment methods described herein. A human having or at risk for developing cochlear hair cell loss can hear less well than the average human being, or less well than a human before

experiencing the hair cell loss. For example, hearing can be diminished by at least 5, 10, 30, 50% or more.

The subject can have hearing loss associated with cochlear hair cell loss for any reason, or as a result of any type of event. For example, a subject can be deaf or  
5 hard-of-hearing as a result of a physical ototoxic insult, e.g., a traumatic event, such as a physical trauma to a structure of the ear. In preferred embodiments, the subject can have (or be at risk of developing) hearing loss as result of exposure to a sudden loud noise, or a prolonged exposure to loud noises. For example, prolonged or repeated exposures to concert venues, airport runways, and construction areas can  
10 cause inner ear damage and subsequent hearing loss; subjects who are subjected to high levels of environmental noise, e.g., in the home or workplace, can be treated using the methods described herein. A subject can have a hearing disorder that results from aging, e.g., presbycusis, which is generally associated with normal aging processes; see, e.g., Huang, *Minn Med.* 90(10):48-50 (2007) and Frisina, *Annals of the New York Academy of Sciences*, 1170: 708–717 (2009), and can occur in subjects  
15 as young as 18, but is generally more marked in older subjects, e.g., subjects over age 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90. A subject can have tinnitus (characterized by ringing in the ears) due to loss of hair cells. A subject can experience a chemical ototoxic insult, wherein ototoxins include therapeutic drugs including antineoplastic  
20 agents, salicylates, quinines, and aminoglycoside antibiotics, e.g., as described further below, contaminants in foods or medicinals, and environmental or industrial pollutants.

In some embodiments, the methods include administering to the subject a compound described herein within one, two, three, four, five, six, or seven days, or  
25 one, two, three, four, five, or six weeks of exposure to an ototoxic insult, e.g., a physical (noise, trauma) or chemical (ototoxin) insult that results in or could result in a loss of hair cells, and causes an increase in Notch signaling in the subject.

In some embodiments, a subject suitable for the treatment using the compounds and methods featured in the invention can include a subject having a  
30 vestibular dysfunction, including bilateral and unilateral vestibular dysfunction; the methods include administering a therapeutically effective amount of an agent described herein, e.g., by systemic administration or administration via the endolymphatic sac (ES). Vestibular dysfunction is an inner ear dysfunction

characterized by symptoms that include dizziness, imbalance, vertigo, nausea, and fuzzy vision and may be accompanied by hearing problems, fatigue and changes in cognitive functioning. Vestibular dysfunctions that can be treated by the methods described herein can be the result of a genetic or congenital defect; an infection, such as a viral or bacterial infection; or an injury, such as a traumatic or nontraumatic injury, that results in a loss of vestibular hair cells. In some embodiments, balance disorders or Meniere's disease (idiopathic endolymphatic hydrops) may be treated by the methods described herein. Vestibular dysfunction is most commonly tested by measuring individual symptoms of the disorder (e.g., vertigo, nausea, and fuzzy vision).

Alternatively or in addition, the compounds and methods featured in the invention can be used prophylactically, such as to prevent, reduce or delay progression of hearing loss, deafness, or other auditory disorders associated with loss of hair cells. For example, a composition containing one or more compounds can be administered with (e.g., before, after or concurrently with) an ototoxic therapy, i.e., a therapeutic that has a risk of hair cell toxicity and thus a risk of causing a hearing disorder. Ototoxic drugs include the antibiotics neomycin, kanamycin, amikacin, viomycin, gentamycin, tobramycin, erythromycin, vancomycin, and streptomycin; chemotherapeutics such as cisplatin; nonsteroidal anti-inflammatory drugs (NSAIDs) such as choline magnesium trisalicylate, diclofenac, diflunisal, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenamate, nabumetone, naproxen, oxaprozin, phenylbutazone, piroxicam, salsalate, sulindac, and tolmetin; diuretics; salicylates such as aspirin; and certain malaria treatments such as quinine and chloroquine. For example, a subject undergoing chemotherapy can be treated using the compounds and methods described herein. The chemotherapeutic agent cisplatin, for example, is known to cause hearing loss. Therefore, a composition containing one or more compounds can be administered with cisplatin therapy (e.g., before, after or concurrently with) to prevent or lessen the severity of the cisplatin side effect. Such a composition can be administered before, after and/or simultaneously with the second therapeutic agent. The two agents may be administered by different routes of administration.

In general, the compounds and methods described herein can be used to generate hair cell growth in the ear and/or to increase the number of hair cells in the

ear (e.g., in the inner, middle, and/or outer ear). For example, the number of hair cells in the ear can be increased about 2-, 3-, 4-, 6-, 8-, or 10-fold, or more, as compared to the number of hair cells before treatment. This new hair cell growth can effectively restore or establish at least a partial improvement in the subject's ability to hear. For  
5 example, administration of an agent can improve hearing loss by about 5, 10, 15, 20, 40, 60, 80, 100% or more.

Where appropriate, following treatment, the subject can be tested for an improvement in hearing or in other symptoms related to inner ear disorders. Methods for measuring hearing are well-known and include pure tone audiometry, air  
10 conduction, and bone conduction tests. These exams measure the limits of loudness (intensity) and pitch (frequency) that a subject can hear. Hearing tests in humans include behavioral observation audiometry (for infants to seven months), visual reinforcement orientation audiometry (for children 7 months to 3 years); play audiometry for children older than 3 years; and standard audiometric tests for older  
15 children and adults, e.g., whispered speech, pure tone audiometry; tuning fork tests; brain stem auditory evoked response (BAER) testing or auditory brain stem evoked potential (ABEP) testing. Oto-acoustic emission testing can be used to test the functioning of the cochlear hair cells, and electro-cochleography provides information about the functioning of the cochlea and the first part of the nerve pathway to the  
20 brain. In some embodiments, treatment can be continued with or without modification or can be stopped.

#### *Dosage*

An "effective amount" is an amount sufficient to effect beneficial or desired therapeutic effect. This amount can be the same or different from a prophylactically  
25 effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions can be administered one from one or more times per day to one or  
30 more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other

diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments. In some embodiments, e.g., in subjects exposed to prolonged or repeated exposures to noise, e.g., normal noises such as are associated with activities of daily life (such as lawnmowers, trucks, motorcycles, airplanes, music (e.g., from personal listening devices), sporting events, etc.), or loud noises, e.g., at concert venues, airports, and construction areas, that can cause inner ear damage and subsequent hearing loss; e.g., subjects who are subjected to high levels of environmental noise, e.g., in the home or workplace, can be treated with repeated, e.g., periodic, doses of the pharmaceutical compositions, e.g., to prevent (reduce the risk of) or delay progression or hearing loss.

Dosage, toxicity and therapeutic efficacy of the therapeutic compounds can be determined by standard pharmaceutical procedures, e.g., in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. For example, samples of the perilymph or endolymph can be obtained to evaluate pharmacokinetics and approximate an effective dosage, e.g., in animal models, e.g., after administration to the round window. The dosage of such compounds lies preferably within a range of concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated from cell culture assays, and/or a dose may be formulated in animal models; alternatively, for those compounds that have been previously used in humans, clinically desirable concentrations can be used as a

starting point. Such information can be used to more accurately determine useful doses in humans.

*Pharmaceutical Compositions and Methods of Administration*

The methods described herein include the manufacture and use of  
5 pharmaceutical compositions that include compounds identified herein, e.g., Notch inhibitors, e.g., gamma-secretase inhibitors, as active ingredients. Also included are the pharmaceutical compositions themselves.

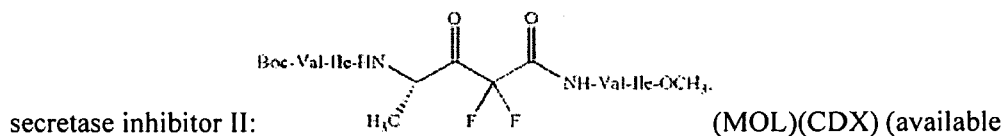
The compositions include one or more notch inhibitors, e.g., gamma secretase inhibitors, e.g., RO4929097; DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-  
10 phenyl]glycine-1,1-dimethylethyl ester); L-685458 ((5S)-(t-Butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl)-L-leu-L-phe-amide); BMS-708163 (Avagacestat); BMS-299897 (2-[(1R)-1-[[[(4-Chlorophenyl)sulfonyl](2,5-difluorophenyl)amino]ethyl-5-fluorobenzenebutanoic acid); MK-0752; YO-01027; MDL28170 (Sigma); LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-  
15 hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide, see US 6,541,466); ELN-46719 (2-hydroxy-valeric acid amide analog of LY411575 (where LY411575 is the 3,5-difluoro-mandelic acid amide) (US Patent No 6,541,466)); PF-03084014 ((S)-2-((S)-5,7-difluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-N-(1-(2-methyl-1-(neopentylamino)propan-2-yl)-  
20 1H-imidazol-4-yl)pentanamide, Samon et al., Mol Cancer Ther 2012;11:1565-1575); and Compound E ((2S)-2-[[[(3,5-Difluorophenyl)acetyl]amino]-N-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl]propanamide; see WO 98/28268 and Samon et al., Mol Cancer Ther 2012;11:1565-1575; available from Alexis Biochemicals)), or pharmaceutically acceptable salts thereof.

25 In some embodiments, suitable gamma secretase inhibitors include: semagacestat (also known as LY450139, (2S)-2-hydroxy-3-methyl-N-[(1S)-1-methyl-2-oxo-2-[[[(1S)-2,3,4,5-tetrahydro-3-methyl-2-oxo-1H-3-benzazepin-1-yl]amino]ethyl]butanamide, available from Eli Lilly; WO 02/47671 and U.S. Pat. No. 7,468,365); LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-  
30 ((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-L-alaninamide, available from Eli Lilly, Fauq et al., Bioorg Med Chem Lett 17: 6392-5, 2007); begacestat (also known as GSI-953, U.S. Pat. No. 7,300,951); arylsulfonamides (AS, Fuwa et al., Bioorg Med Chem Lett. 16(16):4184-4189, 2006); N-[N-(3,5-

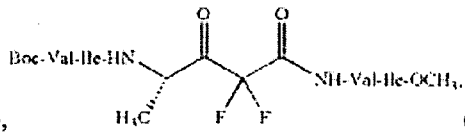
difluorophenacetyl)-L-alanyl]-S)-phenylglycine t-butyl ester (DAPT, Shih and Wang, *Cancer Res.* 67: 1879-1882, 2007); N-[N-3,5-Difluorophenacetyl]-L-alanyl-S-phenylglycine Methyl Ester (also known as DAPM, gamma-Secretase Inhibitor XVI, available from EMD Millipore); Compound W (3,5-bis(4-Nitrophenoxy)benzoic acid, available from Tocris Bioscience); L-685,458 ((5S)-(tert-Butoxycarbonylamino)-6-phenyl-(4R)-hydroxy-(2R)-benzylhexanoyl)-L-leucyl-L-phenylalaninamide, available from Sigma-Aldrich, Shearmen et al., *Biochemistry* 39, 8698-8704, 2000); BMS-289948 (4-chloro-N-(2,5-difluorophenyl)-N-((1R)-{4-fluoro-2-[3-(1H-imidazol-1-yl)propyl]phenyl}ethyl)benzenesulfonamide hydrochloride, available from Bristol Myers Squibb); BMS-299897 (4-[2-((1R)-1-[[4-chlorophenyl)sulfonyl]-2,5-difluoroanilino]ethyl)-5-fluorophenyl]butanoic acid, available from Bristol Myers Squibb, see Zheng et al., *Xenobiotica* 39(7):544-55, 2009); avagacestat (also known as BMS-708163, (R)-2-(4-chloro-N-(2-fluoro-4-(1,2,4-oxadiazol-3-yl)benzyl)phenylsulfonamido)-5,5,5-trifluoropentanamide, available from Bristol Myers Squibb, Albright et al., *J Pharmacol. Exp. Ther.* 344(3):686-695, 2013); MK-0752 (3-(4-((4-chlorophenyl)sulfonyl)-4-(2,5-difluorophenyl)cyclohexyl)propanoic acid, available from Merck); MRK-003 ((3'R,6R,9R)-5'-(2,2,2-trifluoroethyl)-2-((E)-3-(4-(trifluoromethyl)piperidin-1-yl)prop-1-en-1-yl)-5,6,7,8,9,10-hexahydrospiro[6,9-methanobenzo[8]annulene-11,3'-[1,2,5]thiadiazolidine] 1',1'-dioxide, available from Merck, Mizuma et al., *Mol Cancer Ther.* 11(9):1999-2009, 2012); MRK-560 (N-[cis-4-[(4-Chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoro-methanesulfonamide, Best et. al., *J Pharmacol Exp Ther.* 317(2):786-90, 2006); RO-4929097 (also known as R4733, (S)-2,2-dimethyl-N1-(6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-N3-(2,2,3,3,3-pentafluoropropyl)malonamide, available from Hoffman-La Roche Inc., Tolcher et al., *J Clin. Oncol.* 30(19):2348-2353, 2012); JLK6 (also known as 7-Amino-4-chloro-3-methoxyisocoumarin, available from Santa Cruz Biotechnology, Inc., Petit et al., *Nat. Cell. Biol.* 3: 507-511, 2001); Tarenflurbil (also known as (R)-Flurbiprofen, (2R)-2-(3-fluoro-4-phenylphenyl)propanoic acid); ALX-260-127 (also known as Compound 11, described by Wolfe et al., *J. Med. Chem.* 41: 6, 1998); Sulindac sulfide (SSide, Takahashi et al., *J Biol Chem.* 278(20): 18664-70, 2003); 1,1,1-trifluoro-N-(4-[5-fluoro-2-(trifluoromethyl)phenyl]-4-[[4-(trifluoromethyl)phenyl]sulfonyl]cyclohexyl)methanesulfonamide (described in US20110275719); N-[trans-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-

difluorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2-cyano-5-fluorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-dichlorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-(cis-3-(2,5-difluorophenyl)-3-[[4-(trifluoromethyl)phenyl]sulfonyl]cyclobutyl)-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-{cis-3-(5-chloro-2-fluorophenyl)-3-[(4-chlorophenyl)sulfonyl]cyclobutyl}-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-{cis-3-(2,5-difluorophenyl)-3-[(4-fluorophenyl)sulfonyl]cyclobutyl}-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-{cis-3-(2,5-difluorophenyl)-3-[(3,4-difluorophenyl)sulfonyl]cyclobutyl}-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-[cis-3-[(4-cyanophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);4-[[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl][trifluoromethyl]sulfonyl]amino}butanoic acid (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoro-N-[2-(tetrahydro-2-pyran-2-yloxy)ethyl]methanesulfonamide (described in US20110263580);Methyl{[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl][(trifluoromethyl)sulfonyl]amino}acetate (described in US20110263580);N-[3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoro-N-methylmethanesulfonamide (described in US20110263580);N-[3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoro-N-methylmethanesulfonamide (described in US20110263580);Methyl 4-[[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl][(trifluoromethyl)sulfonyl]amino}butanoate (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-N-[[trifluoromethyl]sulfonyl]glycine (described in US20110263580);N-[cis-3-[(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)-1-methylcyclobutyl]-1,1,1-

trifluoromethanesulfonamide (described in US20110263580);N-(cis-3-(2,5-difluorophenyl)-1-methyl-3-{[4-(trifluoromethyl)phenyl]sulfonyl}cyclobutyl)-1,1,1-trifluoromethanesulfonamide (described in US20110263580);N-[cis-3-(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoro-N-[(trifluoromethyl)sulfonyl]methanesulfonamide (described in US20110263580);Sodium[cis-3-(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl][(trifluoromethyl)sulfonyl]azanide (described in US20110263580);Potassium[cis-3-(4-chlorophenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl][(trifluoromethyl)sulfonyl]azanide (described in US20110263580);N-[cis-3-(4-trifluoromethoxyphenyl)sulfonyl]-3-(2,5-difluorophenyl)cyclobutyl]-1,1,1-trifluoromethanesulfonamide (described in US20110263580);1,1,1-trifluoro-N-(4-[5-fluoro-2-(trifluoromethyl)phenyl]-4-{[4-(trifluoromethyl)phenyl]sulfonyl}cyclohexyl)methanesulfonamide (described in US20110263580);gamma-Secretase Inhibitor I (also known as Z-Leu-Leu-Nle-CHO, benzyloxycarbonyl-leucyl-leucyl-norleucinal, available from Calbiochem);gamma-



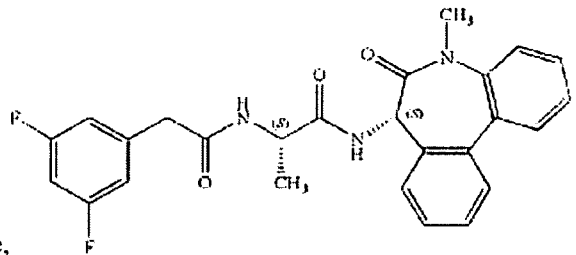
secretase inhibitor III, (N-Benzyloxycarbonyl-Leu-leucinal, available from Calbiochem);gamma secretase inhibitor IV, (N-(2-Naphthoyl)-Val-phenylalaninal, available from Calbiochem);gamma-secretase inhibitor V (also known as Z-LF-CHO, N-Benzyloxycarbonyl-Leu-phenylalaninal, available from EMD Millipore);gamma-secretase inhibitor VI (1-(S)-endo-N-(1,3,3)-Trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl Sulfonamide, available from EMD Millipore);gamma secretase inhibitor VII, (also known as Compound A, MOC-LL-CHO, Menthyloxycarbonyl-LL-CHO, available from Calbiochem);gamma secretase inhibitor X, ({1S-Benzyl-4R-[1-(1S-carbamoyl-2-phenethylcarbamoyl)-1S-3-methylbutylcarbamoyl]-2R-hydroxy-5-phenylpentyl}carbamic acid tert-butyl ester, available from Calbiochem);gamma secretase inhibitor XI, (7-Amino-4-chloro-3-methoxyisocoumarin, available from Calbiochem);gamma secretase inhibitor XII, (also known as Z-Ile-Leu-CHO, Shih and Wang, Cancer Res. 67: 1879-1882, 2007);gamma secretase inhibitor XIII, (Z-Tyr-Ile-Leu-CHO, available from Calbiochem);gamma secretase inhibitor XIV, (Z-Cys(t-Bu)-Ile-Leu-CHO, available from Calbiochem);gamma secretase inhibitor XVII, (also known as WPE-III-



31C), (MOL)(CDX) (available from

Calbiochem);gamma secretase inhibitor XIX, (also known as benzodiazepine, (2S,3R)-3-(3,4-Difluorophenyl)-2-(4-fluorophenyl)-4-hydroxy-N-((3S)-2-oxo-5-

5 phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-butyramide, Churcher et al., J Med Chem. 46(12):2275-8, 2003);gamma secretase inhibitor XX, (also known as dibenzazepine, (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]-N-(5-methyl-6-oxo-6,7-



yl)propionamide, (MOL)(CDX)

(Weihofen et al., Science 296: 2215-2218, 2002, available from Calbiochem);gamma  
 10 secretase inhibitor XXI, ((S,S)-2-[2-(3,5-Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide, available from Calbiochem);5-methyl-2-propan-2-ylcyclohexylN-[4-methyl-1-[(4-methyl-1-oxopentan-2-yl)amino]-1-oxopentan-2-yl]carbamate (available from HDH Pharma Inc.);N-trans-3,5-Dimethoxycinnamoyl-Ile-leucinal (available from Calbiochem);N-tert-Butyloxycarbonyl-Gly-Val-Valinal; isovaleryl-V V-Sta-A-Sta-OCH3 (available from Calbiochem);diethyl-(5-phenyl-3H-azepin-2-yl)-amine (described in US  
 15 8188069);diethyl-(5-isopropyl-3H-azepin-2-yl)-amine (described in US 8188069);diethyl-(4-phenyl-3H-azepin-2-yl)-amine (described in US 8188069); diethyl-(6-phenyl-3H-azepin-2-yl)-amine (described in US 8188069);5-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);5-Isopropyl-1,3-dihydro-azepin-2-one (described in US 8188069);4-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);6-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);2-butoxy-5-phenyl-3H-azepine (described in US 8188069);1-methyl-5-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);5-isopropyl-1-methyl-1,3-dihydro-azepin-2-one  
 20 (described in US 8188069);1-methyl-4-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);1-methyl-6-phenyl-1,3-dihydro-azepin-2-one (described in US 8188069);1-methyl-5-phenyl-1H-azepine-2,3-dione-3-oxime (described in US

8188069);5-isopropyl-1-methyl-1H-azepine-2,3-dione-3-oxime (described in US  
8188069);1-methyl-6-phenyl-1H-azepine-2,3-dione-3-oxime (described in US  
8188069);1-methyl-4-phenyl-1H-azepine-2,3-dione-3-oxime (described in US  
8188069);3-amino-1-methyl-5-phenyl-1,3-dihydro-azepin-2-one (described in US  
5 8188069);3-amino-5-isopropyl-1-methyl-1,3-dihydro-azepin-2-one (described in US  
8188069);3-amino-1-methyl-4-phenyl-1,3-dihydro-azepin-2-one (described in US  
8188069);3-amino-1-methyl-6-phenyl-1,3-dihydro-azepin-2-one (described in US  
8188069);(S)-[1-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoyl)-  
ethyl]-carbamic acid tertbutyl ester (described in US 8188069);[(S)-1-(5-isopropyl-1-  
10 methyl-2-oxo-2,3-dihydro-1H-azepin-3-ylcarbamoyl)-ethyl]carbamic acid tert-butyl  
ester (described in US 8188069);[(S)-1-(1-methyl-2-oxo-4-phenyl-2,3-dihydro-1H-  
azepin-3-ylcarbamoyl)-ethyl]carbamic acid tert-butyl ester (described in US  
8188069);[(S)-1-(1-methyl-2-oxo-6-phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoyl)-  
ethyl]-carbamic acid tert-butyl ester (described in US 8188069);(S)-2-amino-N-(1-  
15 methyl-2-oxo-5-phenyl-2,3-dihydro-1H-azepin-3-yl)-propionamide (described in US  
8188069);(S)-2-amino-N-(5-isopropyl-1-methyl-2-oxo-2,3-dihydro-1H-azepin-3-  
yl)propionamide (described in US 8188069);(S)-2-Amino-N-(1-methyl-2-oxo-6-  
phenyl-2,3-dihydro-1H-azepin-3-yl)propionamide hydrochloride (described in US  
8188069);(S)-2-Amino-N-(1-methyl-2-oxo-4-phenyl-2,3-dihydro-1 H -azepin-3-  
20 yl)propionamide hydrochloride (described in US 8188069);(S)-2-fluoro-3-methyl-  
butyric acid (described in US 8188069);(S)-2-hydroxy-3-methyl-N-[(S)-1-((S)-1-  
methyl-2-oxo-5-phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoyl)-ethyl]-butyramide  
(described in US 8188069);(S)-2-fluoro-3-methyl-N-[(S)-1-(1-methyl-2-oxo-5-  
phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoyl)-ethyl]-butyramide (described in US  
25 8188069);(S)-2-hydroxy-N-[(S)-1-(5-isopropyl-1-methyl-2-oxo-2,3-dihydro-1H-  
azepin-3-ylcarbamoylethyl]-3-methyl-butylamide (described in US 8188069);(S)-2-  
hydroxy-3-methyl-N-[(S)-1-(1-methyl-2-oxo-4-phenyl-2,3-dihydro-1H-azepin-3-  
ylcarbamoylethyl)-butylamide (described in US 8188069);(S)-2-hydroxy-3-methyl-  
N-[(S)-1-(1-methyl-2-oxo-6-phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoylethyl)-  
30 butylamide (described in US 8188069); and(S)-2-fluoro-3-methyl-N-[(S)-1-(1-  
methyl-2-oxo-6-phenyl-2,3-dihydro-1H-azepin-3-ylcarbamoylethyl)-butylamide  
(described in US 8188069), or pharmaceutically acceptable salts thereof.

Additional examples of gamma-secretase inhibitors are disclosed in U.S. Patent Application Publication Nos. 2004/0029862, 2004/0049038, 2004/0186147, 2005/0215602, 2005/0182111, 2005/0182109, 2005/0143369, 2005/0119293, 2008/008316, and 2011/0020232, and U.S. Pat. Nos. 6,756,511; 6,890,956; 5 6,984,626; 7,049,296; 7,101,895; 7,138,400; 7,144,910; 7,183,303; 8,188,069; and International Publication Nos. WO 1998/28268; WO 2001/70677, WO 2002/049038, WO 2004/186147, WO 2003/093253, WO 2003/093251, WO 2003/093252, WO 2003/093264, WO 2005/030731, WO 2005/014553, WO 2004/039800, WO 2004/039370, EP2244713.

10 The entire disclosures of all of the foregoing are hereby incorporated by reference herein.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, 15 isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions, e.g., dexamethasone; prednisone; gentamicin; brain-derived neurotrophic factor (BDNF); recombinant human insulin-like growth factor 1 (rhIGF-1), FGF, R-spondin, and/or GSK-3 $\beta$  antagonists or inhibitors, e.g., one or more of 20 the following GSK3 $\beta$  inhibitors: Purvalanol A, olomoucine; lithium chloride (LiCl), alsterpaullone, and kenpaullone. Other GSK3 $\beta$ -inhibitors that are useful in the treatments described herein include benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8); 2-thio(3-iodobenzyl)-5-(1-pyridyl)-[1,3,4]-oxadiazole (GSK3 inhibitor II); 2,4-dibenzyl-5-oxothiadiazolidine-3-thione (OTDZT); (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO);  $\alpha$ -4-Dibromoacetophenone (i.e., Tau Protein Kinase I (TPK I) 25 Inhibitor), 2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone, N-(4-Methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418), H-KEAPPAPPQSpP-NH<sub>2</sub> (L803; SEQ ID NO:1); Myr-N-GKEAPPAPPQSpP-NH<sub>2</sub> (L803-mts; SEQ ID NO:2); and indirubins. Exemplary indirubins include indirubin-5-sulfonamide; indirubin-5-sulfonic acid (2-hydroxyethyl)-amide indirubin-3'-monoxime; 5-iodo-indirubin-3'- 30 monoxime; 5-fluoroindirubin; 5, 5'-dibromoindirubin; 5-nitroindirubin; 5-chloroindirubin; 5-methylindirubin; and 5-bromoindirubin. Other GSK3 $\beta$ -inhibitors that can be used are known in the art, e.g., those disclosed in Patent Nos. 6,417,185;

6,489,344; 6,608,063 and Published U.S. Applications Nos. 690497, filed October 20, 2003; 468605, filed August 19, 2003; 646625, filed August 21, 2003; 360535, filed February 6, 2003; 447031, filed May 28, 2003; and 309535 filed December 3, 2002.

The present pharmaceutical compositions are formulated to be compatible  
5 with the intended route of administration.

In some embodiments, the compositions are delivered systemically, e.g., by parenteral, e.g., intravenous, intradermal, or subcutaneous administration.

In some embodiments, the compositions are administered by application of a liquid or gel formulation to the round window membrane. Application to the round  
10 window membrane can be accomplished using methods known in the art, e.g., intra-tympanic injection of a liquid or gel formulation or by direct delivery into the inner ear fluids, e.g., using a microfluidic device.

In some embodiments, the compositions are delivered via a pump, e.g., a mini-osmotic pump, see, e.g., Takemura et al., *Hear Res.* 2004 Oct;196(1-2):58-68, or a  
15 catheter, see, e.g., Charabi et al., *Acta Otolaryngol Suppl.* 2000;543:108-10.

Methods of formulating suitable pharmaceutical compositions are known in the art, see, e.g., Remington: The Science and Practice of Pharmacy, 21st ed., 2005; and the books in the series Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs (Dekker, NY). For example, solutions or suspensions  
20 used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as  
25 ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

30 Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline,

bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of  
5 microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of  
10 dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the  
15 injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.  
20 Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yield a powder of the active ingredient plus  
25 any additional desired ingredient from a previously sterile-filtered solution thereof.

In some embodiments, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Liposomal suspensions (including liposomes targeted to selected  
30 cells with monoclonal antibodies to cellular antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. Nanoparticles, e.g., poly lactic/glycolic acid (PLGA) nanoparticles (see

Tamura et al., *Laryngoscope*. 2005 Nov;115(11):2000-5; Ge et al., *Otolaryngol Head Neck Surg*. 2007 Oct;137(4):619-23; Horie et al., *Laryngoscope*. 2010 Feb;120(2):377-83; Sakamoto et al., *Acta Otolaryngol Suppl*. 2010 Nov;(563):101-4) can also be used.

5           In some embodiments, the carrier comprises a polymer, e.g., a hydrogel, that increases retention of the compound on the round window and provides local and sustained release of the active ingredient. Such polymers and hydrogels are known in the art, see, e.g., Paulson et al., *Laryngoscope*. 2008 Apr;118(4):706-11 (describing a chitosan-glycerophosphate (CGP)-hydrogel based drug delivery system); other  
10 carriers can include thermo-reversible triblock copolymer poloxamer 407 (see, e.g., Wang et al., *Audiol Neurotol*. 2009;14(6):393-401. Epub 2009 Nov 16, and Wang et al., *Laryngoscope*. 2011 Feb;121(2):385-91); poloxamer-based hydrogels such as the one used in OTO-104 (see, e.g., GB2459910; Wang et al., *Audiol Neurotol* 2009;14:393-401; and Piu et al., *Otol Neurotol*. 2011 Jan;32(1):171-9); Pluronic F-  
15 127 (see, e.g., Escobar-Chavez et al., *J Pharm Pharm Sci*. 2006;9(3):339-5); Pluronic F68, F88, or F108; polyoxyethylene-polyoxypropylene triblock copolymer (e.g., a polymer composed of polyoxypropylene and polyoxyethylene, of general formula E106 P70 E106; see GB2459910, US20110319377 and US20100273864); MPEG-PCL diblock copolymers (Hyun et al., *Biomacromolecules*. 2007 Apr;8(4):1093-100. Epub 2007 Feb 28); hyaluronic acid hydrogels (Borden et al., *Audiol Neurotol*. 2011;16(1):1-11); gelfoam cubes (see, e.g., Havenith et al., *Hearing Research*, February 2011; 272(1-2):168-177); and gelatin hydrogels (see, e.g., Inaoka et al., *Acta Otolaryngol*. 2009 Apr;129(4):453-7); other biodegradable, biocompatible  
20 polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Tunable self-assembling hydrogels made from natural amino acids L and D can also be used, e.g., as described in Hauser et al e.g. Ac-LD6-COOH (L) e.g. *Biotechnol Adv*. 2012 May-Jun;30(3):593-603. Such formulations can be prepared using standard techniques, or obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc.

30           The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

## EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

### *Materials and Methods*

5           The following materials and methods were used in the examples set forth herein.

#### *Animals*

For the experiments using inner ear spheres, C57BL/6 (Jackson Labs) or *Math1-nGFP* reporter mouse (19) (a gift from Jane Johnson, University of Texas) of both sexes were used. To create organ of Corti explants with ablated hair cells, *Mos-iCsp3* mice (line 17) (20) were crossed with *Pou4f3-Cre* mice (6) (a gift from Douglas Vetter, Tufts University). For all *in vivo* experiments, we used 4-week-old *Cre* reporter line, *mT/mG* (Jackson Labs), crossed to a *Sox2-CreER* mouse (47) (a gift from Konrad Hochedlinger, Mass General Hospital). After genotyping, double transgenic animals were used for lineage tracing. We used young adult wild-type littermates of the *mT/mG*; *Sox2-CreER* mice to prevent strain effects in the response to noise, which are known to vary depending on background (22, 48). Mice were genotyped by PCR. All protocols were approved by the Institutional Animal Care and Use Committee of Massachusetts Eye and Ear Infirmary or the by the ethics committee of Keio University Union on Laboratory Animal Medicine, in compliance with the Public Health Service policy on humane care and use of laboratory animals.

#### *Isolation of inner ear spheres*

The utricles and cochleae of 1- to 3-d-postnatal mice of both sexes were dissected, and after careful removal of the nerve trunk and mesenchymal tissues, were trypsinized and dissociated. Dissociated cells were centrifuged, and the pellet was resuspended and filtered through a 70  $\mu$ m cell strainer (BD Biosciences Discovery Labware) in DMEM/F12 medium with N2/B27 supplement, EGF (20 ng/ml), IGF1 (50 ng/ml), bFGF (10 ng/ml), and heparan sulfate (50 ng/ml) (Sigma). The single cells were cultured in nonadherent Petri dishes (Greiner Bio-One) to initiate clonal growth of spheres (49). Spheres that formed after 2–3 d in culture were passaged every 4–6 d. The spheres were centrifuged, and the pellet was mechanically dissociated with a pipette tip and resuspended in medium. Passage 3–4 spheres were

used for experiments described here. These cells are negative for hair cell markers (37) before the initiation of differentiation. For differentiation, floating spheres were transferred to fibronectin-coated 4 well plates (Greiner Bio-One) as described before (37, 49). Attached spheres were differentiated for 5–7 d in DMEM/F12 medium with N2/B27 supplement but without growth factors.

Gamma-secretase inhibitors, DAPT, L-685458, MDL28170 (Sigma), and LY411575 (Santa Cruz) (See Fig. 10) were added at several concentrations on the day following cell attachment.

#### *Neonatal cochlear explants*

Cochleae of 3-d-postnatal C57BL/6 or *Mos-iCsp3; Pou4f3-Cre* double transgenic mice of both sexes were dissected in Hanks solution (Invitrogen). To obtain a flat cochlear surface preparation, the spiral ganglion, Reissner's membrane, and the most basal cochlear segment were removed. Explants were plated onto 4 well plates (Greiner Bio-One) coated with poly-L-ornithine (0.01%, Sigma) and laminin (50 µg/ml, Becton Dickinson). Cochlear explants were cultured in DMEM (Invitrogen) with 10% fetal bovine serum. All cultures were maintained in a 5% CO<sub>2</sub>/20% O<sub>2</sub>-humidified incubator (Forma Scientific).

#### *Acoustic overexposure*

4-week-old mice were exposed free-field, awake and unrestrained, in a small reverberant chamber (22). Acoustic trauma was produced by a 2 h exposure to an 8–16 kHz octave band noise presented at 116 dB SPL. The exposure stimulus was generated by a custom white-noise source, filtered (Brickwall Filter with a 60 dB/octave slope), amplified (Crown power amplifier), and delivered (JBL compression driver) through an exponential horn fitted securely to a hole in the top of a reverberant box. Sound exposure levels were measured at four positions within each cage using a 0.25 inch Brüel and Kjær condenser microphone: sound pressure was found to vary by <0.5 dB across these measurement positions.

#### *Systemic or round window administration of LY411575*

4-week-old mice weighing 12 to 16 g were used. Before surgery, the animals were anesthetized with ketamine (20 mg/kg, i.p.) and xylazine (100 mg/kg, i.p.), and

an incision was made posterior to the pinna near the external meatus after local administration of lidocaine (1%). The otic bulla was opened to approach the round window niche. The end of a piece of PE 10 tubing (Becton Dickinson) was drawn to a fine tip in a flame and gently inserted into the round window niche. LY411575 was dissolved in DMSO and diluted 10-fold in polyethylene glycol 400 (Sigma) to a final concentration of 4 mM. This solution (total volume 1  $\mu$ l) was injected into the round window niche of the left ear. Polyethylene glycol 400 with 10% DMSO was injected into the right ear as a control. The solution was administered for 2 min. This approach is presently used clinically (e.g. transtympanic injection of steroids for sudden hearing loss and gentamicin for severe balance disorders) and has the advantage of sparing the inner ear but still taking advantage of the local route provided by the round window membrane for delivery of drug into the inner ear (50). Gelatin was placed on the niche to maintain the solution, and the wound was closed.

For systemic administration, LY411575 (50 mg/kg) dissolved in 0.5% (wt/vol) methylcellulose (WAKO) was injected orally once daily for 5 consecutive d. Hearing was measured by ABR at 1 d before, 2 d, 1, 2 week, 1, 2 and 3 months after noise exposure.

#### *qRT-PCR*

The organs of Corti were dissected in HBSS (Invitrogen) and stored in RNAlater (Ambion) at  $-80^{\circ}$  C until further use. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For reverse transcription, SuperScript II (Invitrogen) was used with random hexamers. The reverse transcription conditions were  $25^{\circ}$  C for 10 min followed by  $37^{\circ}$  C for 60 min. The reaction was terminated at  $95^{\circ}$  C for 5 min. cDNAs were mixed with Taqman Gene Expression Mastermix (Applied Biosystems) and *Hes5*, *Atoh1*, or *18S* primers (Applied Biosystems) according to the manufacturer's instructions. Samples were analyzed in 96 wells in triplicate by qPCR (Applied Biosystems 7900HT), and PCR thermal cycling conditions were as follows: initial denaturation at  $95^{\circ}$  C for 2 min, denaturation at  $95^{\circ}$  C for 15 s, annealing/extension at  $60^{\circ}$  C for 1 min for 45 cycles. Conditions were kept constant for each primer. Each PCR reaction was carried out in triplicate. Relative gene expression was analyzed by using the  $\Delta\Delta C_T$  method. Gene

expression was calculated relative to 18S RNA, and the amount of cDNA applied was adjusted so that the Ct value for 18S RNA was between 8 and 11.

#### *Immunohistochemistry*

- 5 For spheres, cells were fixed for 10 min with 4% paraformaldehyde in PBS. Immunostaining was initiated by blocking for 1 h with 0.1% Triton X-100 in PBS supplemented with 1% BSA and 5% goat serum (PBT1). Fixed and permeabilized cells were incubated overnight in PBT1 with polyclonal antibody to myosin VIIa (Proteus Biosciences). Samples were washed three times for 20 min with PBS.
- 10 Primary antibodies were detected with secondary antibodies conjugated with Alexa 488 (Molecular Probes), with secondary antibody alone used as a negative control. The samples were counterstained with DAPI (Vector Laboratories) or Hoechst 33258 (Invitrogen) for 10 min and viewed by epifluorescence microscopy (Axioskop 2 Mot Axiocam, Zeiss).
- 15 For explants, the organs of Corti were fixed for 15 min with 4% paraformaldehyde in PBS. Immunostaining was initiated by blocking the tissues for 1 h with 0.1% Triton X-100 in PBS supplemented with 5% donkey serum (PBT1). Fixed and permeabilized cells were incubated overnight in PBT1 with polyclonal antibody to myosin VIIa (Proteus Biosciences), Sox2 (Santa Cruz), GFP (Invitrogen),
- 20 prestin (Santa Cruz), and CtBP2 (BD Biosciences). Samples were washed three times for 20 min with PBS. Primary antibodies were detected with secondary antibodies conjugated with Alexa 488 and 647 (Molecular Probes). The samples were stained with rhodamine phalloidin (Invitrogen) for 15 min and viewed by confocal fluorescence microscopy (TCS SP5, Leica).
- 25 For collection of mature mouse cochleae, after being deeply anesthetized with ketamine and xylazine, the mice were transcardially perfused with 0.01 M phosphate buffer (pH 7.4) containing 8.6% sucrose, followed by fixative consisting of freshly depolymerized 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After decapitation, the temporal bones were removed and immediately placed in the same
- 30 fixative at 4° C. Small openings were made at the round window, oval window, and apex of the cochlea. After immersion in the fixative overnight at 4° C, temporal bones were decalcified in 0.1 M EDTA (pH 7.4) containing 5% sucrose with stirring at 4° C for 2 d. After decalcification, cochleae were microdissected into 4 pieces for

whole mount preparation. Immunostaining was initiated by blocking the tissues for 1 h with 0.1% Triton X-100 in PBS supplemented with 5% donkey serum (PBT1). Fixed and permeabilized pieces were incubated overnight in PBT1 with polyclonal antibody to myosin VIIa (Proteus Biosciences), Sox2 (Santa Cruz), and GFP (Invitrogen). Samples were washed three times for 20 min with PBS. Primary antibodies were detected with secondary antibodies conjugated with Alexa 488, 568, and 647 (Molecular Probes) and viewed by confocal fluorescence microscopy (TCS SP5, Leica). Cochlear lengths were obtained for each case, and a cochlear frequency map computed to precisely localize inner hair cells from the 5.6, 8.0, 11.3, 16.0, 22.6, 32, and 45.2 kHz regions. For cross-sectioning, fixed temporal bones were sunk in 30% sucrose in PBS at 4°C, incubated in OCT at room temperature for 1 h, and frozen in liquid nitrogen. The staining protocol was the same as described above except for counterstaining with DAPI (Vector Laboratories). Specimens were viewed by epifluorescence microscopy (Axioskop 2 Mot Axiocam, Zeiss).

#### Cell Counts

Cell counting for spheres was performed with MetaMorph software. The cell number was determined from DAPI- or Hoechst-positive nuclei. Repeat cell counting gave a test variation of <1%. For explants, inner hair cells, outer hair cells, and supporting cells in the outer hair cell region were counted on cochlear whole mounts. Hair cells were identified with myosin VIIa antibodies or endogenous GFP in *Math1-nGFP* mice. High-power images of the full-length cochlea or cochlear explant cultures were assembled and analyzed in PhotoShop CS4 (Adobe). ImageJ software (NIH) was used to measure the total length of cochlear whole mounts and the length of individual counted segments. The total number of inner hair cells, outer hair cells, and supporting cells in the outer hair cell region was counted in each of four cochlear segments of 1200–1400  $\mu\text{m}$  (apical, mid-apical, mid-basal, and basal). Density (cells per 100  $\mu\text{m}$ ) was then calculated for each segment. For mature cochleae, high-power images of frequency-specific regions (5.6, 8.0, 11.3, 16.0 kHz) according to the computed frequency map were assembled and analyzed. The number of inner hair cells, outer hair cells, and supporting cells in the outer hair cell region in 100  $\mu\text{m}$  was counted in each of the four frequency-specific regions of the cochlea. The number of Sox2-lineage-positive cells identified by GFP was counted by the same method.

### *ABR measurements*

Auditory brain stem responses (51, 52) were measured in each animal at 7 log-spaced frequencies (half-octave steps from 5.6 to 45.2 kHz) before and 1 d after noise exposure, and 1-week, 1-month, and 3-months after surgery. Mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (20 mg/kg i.p.). Needle electrodes were inserted at vertex and pinna, with a ground near the tail. ABRs were evoked with 5-ms tone pips (0.5-ms rise-fall with a  $\cos^2$  onset envelope delivered at 35/s). The response was amplified, filtered and averaged in a LabVIEW-driven data-acquisition system. Sound level was raised in 5 dB steps from  $\geq 10$  dB below threshold  $< 80$  dB SPL. At each sound level, 1024 responses were averaged (with stimulus polarity alternated), using an “artifact reject,” whereby response waveforms were discarded when peak-to-peak response amplitude exceeded  $15 \mu\text{V}$ . On visual inspection of stacked waveforms, “ABR threshold” was defined as the lowest SPL level at which any wave could be detected, usually corresponding to the level step just below that at which the peak-to-peak response amplitude rose significantly above the noise floor (approximately  $0.25 \mu\text{V}$ ). When no response was observed at the highest sound level available, the threshold was designated as being 5 dB greater than that level so that statistical tests could be done. For amplitude versus level functions, the wave I peak was identified by visual inspection at each sound level and the peak-to-peak amplitude computed.

### *Quantification and statistical analysis*

The 2-tailed Mann-Whitney U test was used to compare differences among treatment groups. Changes before and after treatment of the same animal were analyzed by 2-tailed Wilcoxon *t* test. Repeated-measures ANOVA was used to compare time-dependent differences among groups. Data are presented in the text and in figures as mean  $\pm$  SEM. P values less than 0.05 were considered significant.

### *Genotyping primers*

*Mos-iCsp3*

LacZ F: 5'-ttcactggccgctcgttttacaacgctgga-3' (SEQ ID NO:1)

LacZ R: 5'-atgtgagcgagtaacaacccgctggattct-3' (SEQ ID NO:2)

*Pou4f3Cre, Sox2CreER*

Cre F: 5'-tgggcggcatggtgcaagtt-3' (SEQ ID NO:3)

Cre R: 5'-cggtgctaaccagcgttttc-3' (SEQ ID NO:4)

5 *mT/mG*

oIMR7318 wild-type F: 5'-ctctgctgcctcctggcttct-3' (SEQ ID NO:5)

oIMR7319 wild-type R: 5'-cgaggcggatcacaagcaata-3' (SEQ ID NO:6)

oIMR7320 mutant R: 5'-tcaatgggcgggggtcgtt-3'(SEQ ID NO:7)

10 *Example 1. Screening for  $\gamma$ -secretase inhibitors that induce hair cell differentiation from inner ear stem cells*

Ligand-triggered  $\gamma$ -secretase activity catalyzes proteolytic release of Notch intracellular domain and thereby mediates the first step of Notch signal transduction. We previously showed that  $\gamma$ -secretase inhibitors promoted hair cell differentiation from inner ear stem cells by an effect on Notch (15). To find the most potent inhibitor several known drugs, DAPT, L-685458, MDL28170, and LY411575 (see Fig. 10), were tested for their effect on hair cell differentiation from utricular spheres derived from neonatal *Math1-nGFP* reporter mice (19). In this system, LY411575 had the highest potency (Fig. 1a) among the four  $\gamma$ -secretase inhibitors. To confirm the effect of LY411575 on cochlear cells, spheres derived from organ of Corti were used. Upon treatment with LY411575, the numbers of myosin VIIa-positive cells (myosin VIIa is a specific marker for hair cells) increased 1.5 to 2.5 fold above control (Fig. 1B). These cells were also positive for calretinin, another marker for hair cells, and their hair bundles were positive for espin.

25 *Example 2. LY411575 increased hair cell number in organ of Corti explants*

The effect of LY411575 was further characterized on neonatal organ of Corti explants. The addition of LY411575 increased the number of myosin VIIa-positive cells in the outer hair cell region (Fig. 1C) by 30 cells/100  $\mu$ m compared to the control (Fig. 1D,  $p < 0.05$ ). The additional hair cells showed hair bundle structures. These results indicated that the  $\gamma$ -secretase inhibitor, which was chosen by screening using inner ear stem cells, effectively induced extra hair cell differentiation in the neonatal organ of Corti.

Next, organ of Corti explants from *Pou4f3-Cre; Mos-iCsp3* double transgenic mice were used to test whether hair cells could be induced after damage (Fig. 2A). This *Mos-iCsp3* mouse has a Cre/lox cassette that produces a drug-regulated dimerizable caspase-3 (20) in hair cells, because *Pou4f3*, which is expressed transiently in the developing inner ear, is limited to hair cells (21). Thus, after treatment with a drug that dimerizes caspase-3, the dimer leads to hair cell death. *Mos-iCsp3* cochleae showed loss of outer hair cells (Fig. 2B vs Fig. 2C, control). LY411575 treatment of *Mos-iCsp3* organ of Corti increased the number of myosin VIIa-positive (hair) cells in the outer hair cell region (Fig. 2D;  $p < 0.05$ ) and was accompanied by a decrease in the number of Sox2-positive (supporting) cells in the mid-apex and mid-base of the cochlea (Fig. 2D;  $p < 0.05$ ). There were no significant differences in the number of inner hair cells in any group. The correlation between the increase in outer hair cells and the decrease in supporting cells after LY411575 treatment suggested that supporting cells transdifferentiated into hair cells when Notch signaling was prevented.

*Example 3. Systemic LY411575 administration increased hair cell number and promoted hearing recovery in a noise-damaged cochlea*

To assess whether hair cell differentiation could be induced in a mature ear, mice were first exposed to an acoustic injury (22) producing widespread outer hair cell death and permanent hearing loss with preservation of supporting cells (see Example 5 and Figs. 7A-C). Oral LY411575 at 50 mg/kg body weight for 5 d decreased the noise-induced threshold shift at 4, 8 and 16 kHz (Fig. 8A). Outer hair cell numbers were increased and the new hair cells had stereociliary bundles (Fig. 8B). The treated mice suffered significant side-effects (see Example 6). A lower dose (10 mg/kg body weight) had no therapeutic benefit.

*Example 4. Local LY411575 administration promoted hearing recovery by supporting cell transdifferentiation into hair cells after noise-induced hearing loss in the mature cochlea*

Due to the dose-limiting toxicity after systemic administration of the drug, direct delivery to the inner ear via the round window membrane, a permeable cellular barrier between the middle and inner ear (23, 24), was tested. The time course of *Hes5* and *Atoh1* mRNA expression levels were assessed in the deafened mature

cochlea in the presence and absence of LY411575 using quantitative RT-PCR. *Hes5* is a direct downstream target of Notch signaling that represses *Atoh1* (25). LY411575 was administered *via* the round window niche 1 d after noise exposure. After the noise exposure, *Hes5* mRNA expression increased by  $2.15 \pm 0.26$  compared to its pre-noise level and its level gradually decreased to reach the pre-noise level 3 d after noise exposure (Fig. 3A). This induction was completely blocked in the LY411575 treated group at 1 d and stayed at the pre-noise level (significant difference from the control cochlea,  $p < 0.01$ ). Three days after LY411575 treatment, the *Hes5* expression level was unchanged from the control cochlea. In contrast to *Hes5*, *Atoh1* expression remained stable after noise exposure (Fig. 3B). Its expression was significantly increased 1 d after LY411575 treatment to 2.28 X above the level post-noise exposure, and remained elevated 3 d after treatment ( $p < 0.05$ ), before returning to the pre-noise level after 7 d. These results showed that a Notch signal could be activated by intense noise trauma, and reduction of *Hes5* in the young adult mouse cochlea by local  $\gamma$ -secretase inhibitor treatment led to sustained upregulation of *Atoh1*.

*In vivo* lineage tracing was used to test whether transdifferentiation could account for new hair cells. A Cre-reporter strain was used to perform lineage tracing of Sox2-positive cells since Sox2 is expressed in supporting cells. In *Sox2-CreER; mT/mG* mice, cells expressing Sox2 at the time of tamoxifen administration become positive for GFP and retain expression even if they lose Sox2 expression (Fig. 9). Reporter mice were exposed to noise 1 week after tamoxifen treatment, and administered LY411575 to the left ear and carrier to the right ear 1 d after noise exposure. One month after LY411575 treatment, numerous myosin VIIa-positive cells in the deafened cochlea also expressed GFP, demonstrating transdifferentiation from Sox2-positive cells. Green hair bundles were observed in the myosin VIIa/GFP double-labeled cells (Fig. 4A and 4C), and some of the bundles appeared in a V-shaped arrangement like the original hair cells (Fig. 4C and D). Furthermore, the GFP-labeled cells showed positive staining for prestin, the motor protein of outer hair cells (Dallos et al., 2006), and were negative for VGLUT3, a marker of inner hair cells (Seal et al., 2008), as well as CtBP2, a synaptic ribbon marker that would be expected to be expressed if the new hair cells were active inner hair cells (Khimich et al., 2005; Liberman et al., 2011). This analysis of markers together with their location

and V-shaped bundles identified them as outer hair cells. These double-labeled cells spanned the epithelium from basilar membrane to the endolymphatic surface (Fig. 4D), which is never seen in the normal ear, but has been reported when supporting cells are transfected with *Atoh1* (16). The nucleus of these cells was at the base of the cell. Double-labeled cells were found in the upper turns of the cochlea, with the highest numbers in the mid apex (Fig. 4G; n = 5). In control ears, no double-labeled cells were observed in any cochlear region (Fig. 4E and F). This result indicated that blocking Notch with LY411575 promoted supporting cell transdifferentiation into hair cells from the apical to mid-apical turn in the mature cochlea after noise-induced hair cell loss.

At 3 months the number of outer hair cells was increased throughout the middle of the cochlea (8 – 16 kHz) in LY411575 treated ears, compared to the carrier-treated contralateral ear (Fig. 5A and B; p < 0.05). The number of supporting cells in the outer hair cell region was decreased significantly in the same cochleae as the increase in outer hair cell number at the 8 and 11.3 kHz areas compared to the carrier-treated ear (Figure 5A and B; p < 0.05). Decreases in supporting cells were also significant (Fig. 5B, p < 0.05) similar to the explant cultures. The outer hair cells were completely absent with and without LY411575 treatment in the most basal regions (above 22 kHz), and there were no significant changes in the numbers of inner hair cells in the treated group (data not shown). The differences in outer hair cell number between LY411575 and carrier-treated ears are larger than the corresponding differences in the number of supporting cells. Furthermore, the differences in outer hair cell number showed a similar trend, in regard to cochlear location, as the myosin VIIa-positive cells from the *Sox2*-lineage observed in *Sox2-CreER; mT/mG* mice (Fig. 4G).

The auditory brainstem response (ABR) was recorded in LY411575 and carrier-treated, control ears to determine the effect of hair cell replacement on the thresholds for a response. Threshold changes were not seen after injection of carrier alone. ABR thresholds 1 d after noise-exposure were >80 dB SPL at all frequencies (Fig. 6A and B). Post-exposure recovery in control ears (Fig. 6A) was minimal under these conditions, as expected (22). Surprisingly, threshold recoveries after LY411575 treatment were significantly greater than control at 8, 11.33 and 16 kHz (Fig. 6D), and wave I amplitudes were increased at the same frequencies (Fig. 6E). No threshold

recoveries were observed in either ear at frequencies above 22.65 kHz by ABR and no recoveries above the noise floor of the distortion product otoacoustic emissions (DPOAE) could be seen. The differences in threshold recovery showed a similar dependence on cochlea location/frequency as outer hair cell number (see Fig. 5).

5 *Example 5. Noise-induced hearing loss in the mature cochlea*

A noise-induced hearing loss model in young adult mice was also used. At an exposure intensity of 116 dB SPL (8-16 kHz), which leads to permanent hearing loss and major hair cell death especially in the outer hair cells region (22), almost all inner hair cells were preserved (from the apical tip to the 22 kHz area), while the outer hair cells showed severe loss. Moreover, almost all supporting cell were preserved (Figs. 7A-C). Thus the 116 dB noise exposure model was selected as a hair cell loss model to investigate hair cell regeneration.

10 *Example 6. Systemic LY411575 treatment ameliorates hearing loss in the mature noise-damaged cochlea*

15 Preliminary range finding experiments for drug treatment were carried out by systemic injection and were limited by toxicity. A minimal dosing regimen for an effect on the thymus weight was chosen (43, 44). Of 12 mice administered 50 mg/kg for 5 d, 6 could be tested for ABR at 3 months, the final time point of the LY411575 treatment. The rest died within the first week due to severe diarrhea and weight loss. Mice that survived also suffered from weight loss (approximately 15% loss in 3 d), with a loss of epithelial cells of their stomach and increase in secreting cells in all gastro-intestinal tract from esophagus to colon and severe atrophy in the spleen in a week; immunosuppression with an atrophy of thymus (total number of the cells were dramatically decreased to 1/40 and double positive fraction of CD4 and CD8 was decreased from 78.6% to 1.23%), changes in the skin color in the next week. Those changes resulted from Notch inhibition reported by previous papers (44, 45). A small threshold shift (Fig. 8A) that achieved statistical significance by comparison of the control and treated animals after 1 month and persisted to 3 months was observed at 4, 8 and 16 kHz. Hair cell counts indicated an increase in outer hair cells, which were most apparent at the regions where the damage was most severe (low frequency, Fig. 8A) and the hair cells appeared to have stereociliary bundles and synapses visualized by double staining with CtBP2 and neurofilament antibody (Fig. 8B).

*Example 7. Model for lineage tracing of supporting cells*

To visualize supporting cell transdifferentiation into hair cells, a reporter line was used: *mT/mG* mice (46) crossed with *Sox2-CreER* mice. In the *mT/mG* mice, cells that have undergone Cre recombination are labeled by expression of membrane-bound GFP (GFP; green fluorescence), and non-recombined cells express td-Tomato (red fluorescence) after tamoxifen treatment. The result is a Cre-reporter line that can be used for lineage tracing of Sox2-positive cells. In the double-transgenic mouse cochlea, after estrogen receptor activation by tamoxifen in Sox2-positive cells, supporting cells expressed green fluorescence from GFP and hair cells retained red fluorescence from td-Tomato (Fig. 9).

REFERENCES

1. A. J. Hudspeth, Making an effort to listen: mechanical amplification in the ear. *Neuron* 59, 530 (Aug 28, 2008).
2. G. D. Nayak, H. S. Ratnayaka, R. J. Goodyear, G. P. Richardson, Development of the hair bundle and mechanotransduction. *Int J Dev Biol* 51, 597 (2007).
3. P. Chen, N. Segil, p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 126, 1581 (Apr, 1999).
4. A. S. Edge, Z. Y. Chen, Hair cell regeneration. *Curr Opin Neurobiol* 18, 377 (Aug, 2008).
5. M. W. Kelley, Regulation of cell fate in the sensory epithelia of the inner ear. *Nat Rev Neurosci* 7, 837 (Nov, 2006).
6. C. Sage et al., Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. *Science* 307, 1114 (Feb 18, 2005).
7. J. T. Corwin, D. A. Cotanche, Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772 (Jun 24, 1988).
8. B. Fritsch, K. W. Beisel, L. A. Hansen, The molecular basis of neurosensory cell formation in ear development: a blueprint for hair cell and sensory neuron regeneration? *Bioessays* 28, 1181 (Dec, 2006).
9. B. M. Ryals, E. W. Rubel, Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. *Science* 240, 1774 (Jun 24, 1988).

10. J. Adam et al., Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* 125, 4645 (Dec, 1998).
11. N. Daudet, J. Lewis, Two contrasting roles for Notch activity in chick  
5 inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* 132, 541 (Feb, 2005).
12. S. A. Batts, C. R. Shoemaker, Y. Raphael, Notch signaling and Hes labeling in the normal and drug-damaged organ of Corti. *Hear Res* 249, 15 (Mar, 2009).
- 10 13. A. Doetzlhofer et al., Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. *Dev Cell* 16, 58 (Jan, 2009).
14. B. H. Hartman et al., Hes5 expression in the postnatal and adult mouse inner ear and the drug-damaged cochlea. *J Assoc Res Otolaryngol* 10, 321 (Sep,  
15 2009).
15. S. J. Jeon, M. Fujioka, S. C. Kim, A. S. B. Edge, Notch signaling alters sensory or neuronal cell fate specification of inner ear stem cells. *J. Neurosci.* 31, 8351 (June 8, 2011).
16. M. Izumikawa et al., Auditory hair cell replacement and hearing  
20 improvement by *Atoh1* gene therapy in deaf mammals. *Nat Med* 11, 271 (Mar, 2005).
17. S. P. Gubbels, D. W. Woessner, J. C. Mitchell, A. J. Ricci, J. V. Brigande, Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature* 455, 537 (Sep 25, 2008).
18. J. L. Zheng, W. Q. Gao, Overexpression of *Math1* induces robust  
25 production of extra hair cells in postnatal rat inner ears. *Nat Neurosci* 3, 580 (Jun, 2000).
19. E. A. Lumpkin et al., *Math1*-driven GFP expression in the developing nervous system of transgenic mice. *Gene Expr Patterns* 3, 389 (Aug, 2003).
20. M. Fujioka, H. Tokano, K. S. Fujioka, H. Okano, A. S. Edge,  
30 Generating mouse models of degenerative diseases using Cre/lox-mediated in vivo mosaic cell ablation. *J Clin Invest* 121, 2462 (Jun 1, 2011).

21. C. Sage et al., Essential role of retinoblastoma protein in mammalian hair cell development and hearing. *Proc Natl Acad Sci U S A* 103, 7345 (May 9, 2006).
22. Y. Wang, K. Hirose, M. C. Liberman, Dynamics of noise-induced cellular injury and repair in the mouse cochlea. *J Assoc Res Otolaryngol* 3, 248 (Sep, 2002).
23. M. V. Goycoolea, L. Lundman, Round window membrane. Structure function and permeability: a review. *Microsc Res Tech* 36, 201 (Feb 1, 1997).
24. A. N. Salt, S. K. Plontke, Principles of local drug delivery to the inner ear. *Audiol Neurootol* 14, 350 (2009).
25. A. Zine et al., Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J Neurosci* 21, 4712 (Jul 1, 2001).
26. P. M. White, A. Doetzlhofer, Y. S. Lee, A. K. Groves, N. Segil, Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 441, 984 (Jun 22, 2006).
27. H. Lowenheim et al., Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. *Proc Natl Acad Sci U S A* 96, 4084 (Mar 30, 1999).
28. J. Mantela et al., The retinoblastoma gene pathway regulates the postmitotic state of hair cells of the mouse inner ear. *Development* 132, 2377 (May, 2005).
29. M. Cohen-Salmon et al., Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. *Curr Biol* 12, 1106 (Jul 9, 2002).
30. A. B. Elgoyhen, L. F. Franchini, Prestin and the cholinergic receptor of hair cells: positively-selected proteins in mammals. *Hear Res* 273, 100 (Mar, 2011).
31. G. P. Richardson, J. B. de Monvel, C. Petit, How the genetics of deafness illuminates auditory physiology. *Annu Rev Physiol* 73, 311 (Mar 17, 2011).
32. M. Caiazzo et al., Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. *Nature* 476, 224 (Aug 11, 2011).
33. T. Vierbuchen et al., Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035 (Feb 25, 2010).

34. Y. Kaneko et al., Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev Neurosci* 22, 139 (2000).
35. E. C. Oesterle, S. Campbell, R. R. Taylor, A. Forge, C. R. Hume, Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J Assoc Res Otolaryngol* 9, 65 (Mar, 2008).
36. H. Sakaguchi et al., Spatiotemporal patterns of Musashi1 expression during inner ear development. *Neuroreport* 15, 997 (Apr 29, 2004).
37. K. Oshima et al., Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. *J Assoc Res Otolaryngol* 8, 18 (Mar, 2007).
38. J. S. Stone, D. A. Cotanche, Hair cell regeneration in the avian auditory epithelium. *Int J Dev Biol* 51, 633 (2007).
39. M. E. Warchol, Sensory regeneration in the vertebrate inner ear: Differences at the levels of cells and species. *Hear Res*, (May 19, 2010).
40. J. Cafaro, G. S. Lee, J. S. Stone, Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev Dyn* 236, 156 (Jan, 2007).
41. N. Daudet et al., Notch regulation of progenitor cell behavior in quiescent and regenerating auditory epithelium of mature birds. *Dev Biol*, (Nov 5, 2008).
42. V. Lin et al., Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J Neurosci* 31, 15329 (Oct 26, 2011).
43. L. A. Hyde et al., Studies to investigate the in vivo therapeutic window of the gamma-secretase inhibitor N2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide (LY411,575) in the CRND8 mouse. *J Pharmacol Exp Ther* 319, 1133 (Dec, 2006).
44. G. T. Wong et al., Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem* 279, 12876 (Mar 26, 2004).
45. B. K. Hadland et al., Gamma -secretase inhibitors repress thymocyte development. *Proc Natl Acad Sci U S A* 98, 7487 (Jun 19, 2001).

46. M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse. *Genesis* 45, 593 (Sep, 2007).

47. K. Arnold et al., Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell* 9, 317 (Oct 4, 2011).

5 48. G. W. Harding, B. A. Bohne, J. D. Vos, The effect of an age-related hearing loss gene (Ahl) on noise-induced hearing loss and cochlear damage from low-frequency noise. *Hear Res* 204, 90 (Jun, 2005).

49. R. Martinez-Monedero, E. Yi, K. Oshima, E. Glowatzki, A. S. Edge, Differentiation of inner ear stem cells to functional sensory neurons. *Dev Neurobiol* 10 68, 669 (Apr, 2008).

50. A. A. Mikulec, J. J. Hartsock, A. N. Salt, Permeability of the round window membrane is influenced by the composition of applied drug solutions and by common surgical procedures. *Otol Neurotol* 29, 1020 (Oct, 2008).

51. S. G. Kujawa, M. C. Liberman, Conditioning-related protection from acoustic injury: effects of chronic deafferentation and sham surgery. *J Neurophysiol* 15 78, 3095 (Dec, 1997).

52. S. F. Maison, R. B. Emeson, J. C. Adams, A. E. Luebke, M. C. Liberman, Loss of alpha CGRP reduces sound-evoked activity in the cochlear nerve. *J Neurophysiol* 90, 2941 (Nov, 2003).

## 20 OTHER EMBODIMENTS

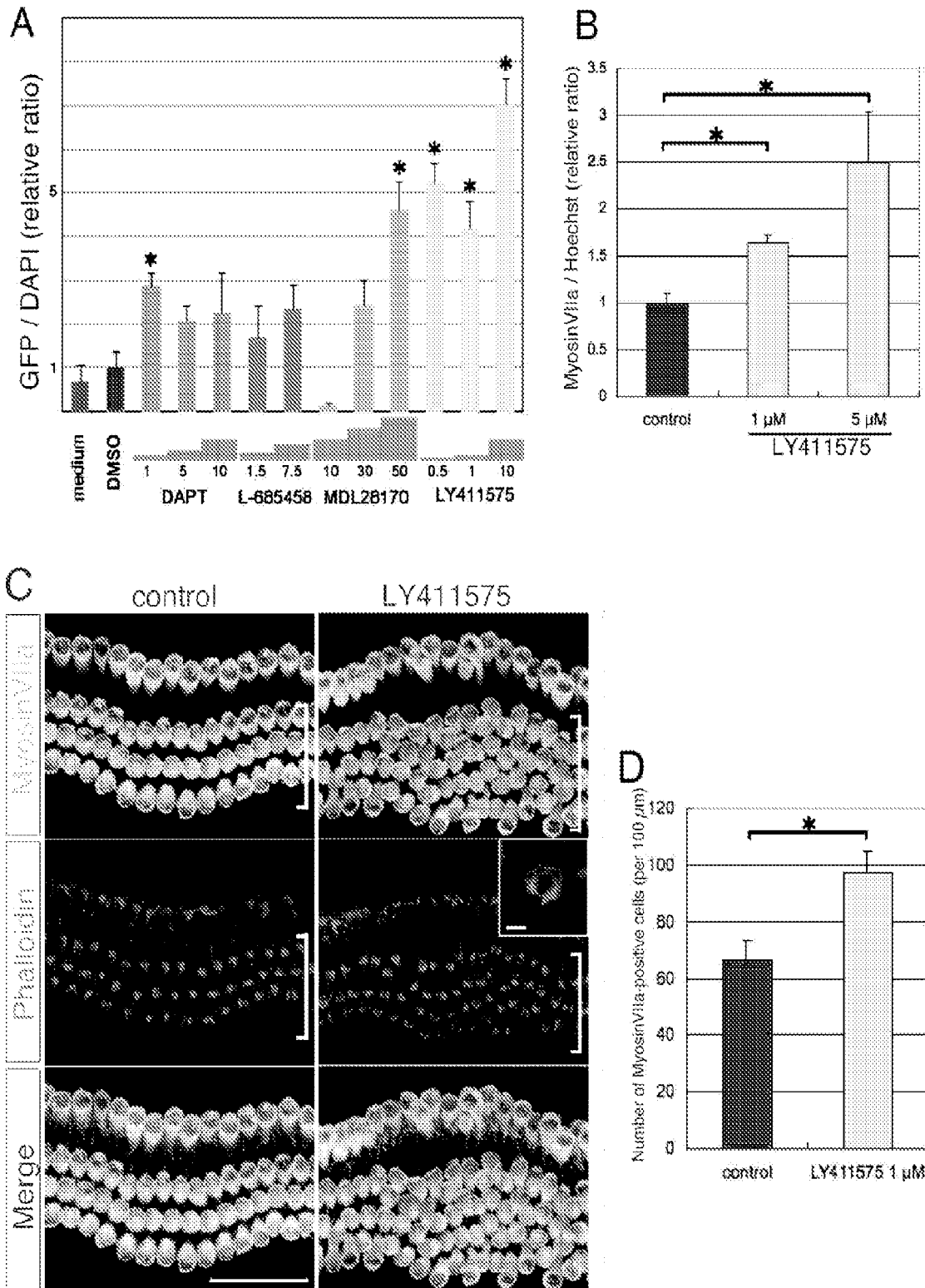
It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the 25 scope of the following claims.

**WHAT IS CLAIMED IS:**

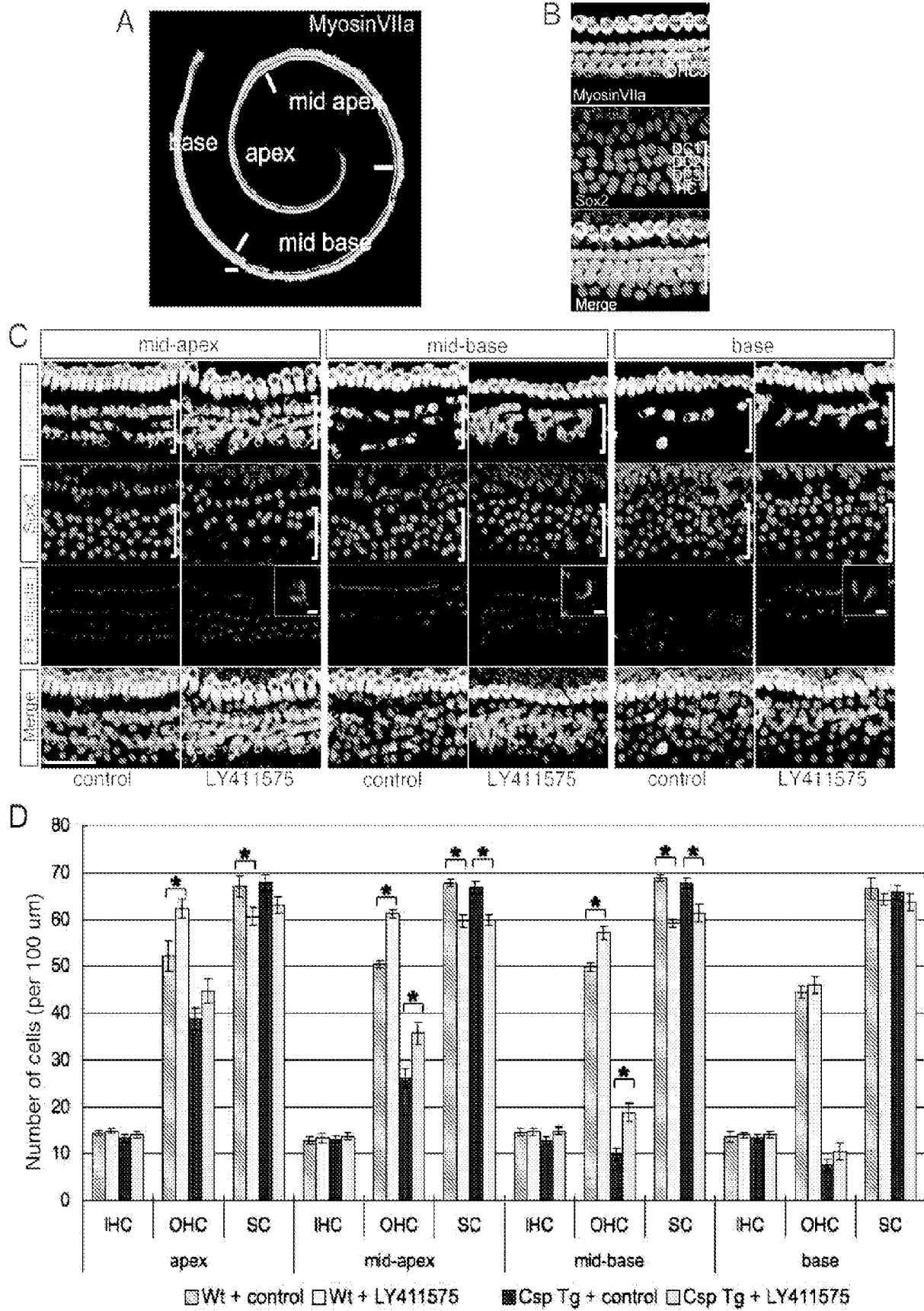
1. A method of treating hearing loss caused by loss of cochlear hair cells in a post-neonatal mammal, the method comprising locally administering to the ear of the mammal a composition comprising a therapeutically effective amount of a Notch inhibitor, e.g., a gamma secretase inhibitor, wherein the therapeutically effective amount is an amount sufficient to restore hearing at one or more frequencies.
2. The method of claim 1, wherein the hearing loss was caused by exposure to a physical or chemical ototoxic insult.
3. The method of claim 2, wherein the physical ototoxic insult is noise.
4. The method of claim 2, wherein the composition is administered to the ear within four weeks, two weeks, one week, or one day of the exposure to the insult.
5. The method of claim 1, wherein the composition is applied topically to the round window.
6. The method of claim 1, wherein the composition further comprises a sustained release carrier.
7. The method of claim 6, wherein the carrier is a polyoxyethylene-polyoxypropylene triblock copolymer.
8. The method of claim 1, wherein the composition comprises at least 10 mM of the Notch inhibitor.
9. The method of claim 1, further comprising determining a baseline level of hearing at one or more frequencies before administering the composition, and a subsequent level of hearing at the same one or more frequencies after administering the composition, and administering one or more additional doses of the composition until a desired level of hearing at the one or more frequencies is recovered.

10. The method of claim 9, wherein the subsequent level of hearing is determined one week, two weeks, three weeks, one month, two months, three months, four months, six months, and/or twelve months after administering the composition.
11. The method of any of the preceding claims, wherein the gamma secretase inhibitor is selected from the group consisting of RO4929097; DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester); L-685458 ((5S)-(t-Butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl)-L-leu-L-phe-amide); BMS-708163 (Avagacestat); BMS-299897 (2-[(1R)-1-[(4-Chlorophenyl)sulfonyl](2,5-difluorophenyl)amino]ethyl-5-fluorobenzenebutanoic acid); MK-0752; YO-01027; MDL28170 (Sigma); LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide); ELN-46719 (2-hydroxy-valeric acid amide analog of LY411575; PF-03084014 ((S)-2-((S)-5,7-difluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-N-(1-(2-methyl-1-(neopentylamino)propan-2-yl)-1H-imidazol-4-yl)pentanamide); Compound E ( (2S)-2-[(3,5-Difluorophenyl)acetyl]amino}-N-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl]propanamide; and Semagacestat (LY450139; (2S)-2-hydroxy-3-methyl-N-((1S)-1-methyl-2-[(1S)-3-methyl-2-oxo-2,3,4,5-tetrahydro-1H-3-benzazepin-1-yl]amino}-2-oxoethyl)butanamide), or pharmaceutically acceptable salts thereof.
12. The method of any of the preceding claims, wherein the gamma secretase inhibitor is LY411575 (N-2((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-l-alaninamide).
13. The method of any of the preceding claims, wherein the mammal is a child, adolescent or adult, e.g., above the age of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 years.
14. The method of any of the preceding claims, wherein the mammal is an adult of at least 40 years of age, e.g., at least 45, 50, 55, 60, 65, 70 years of age.

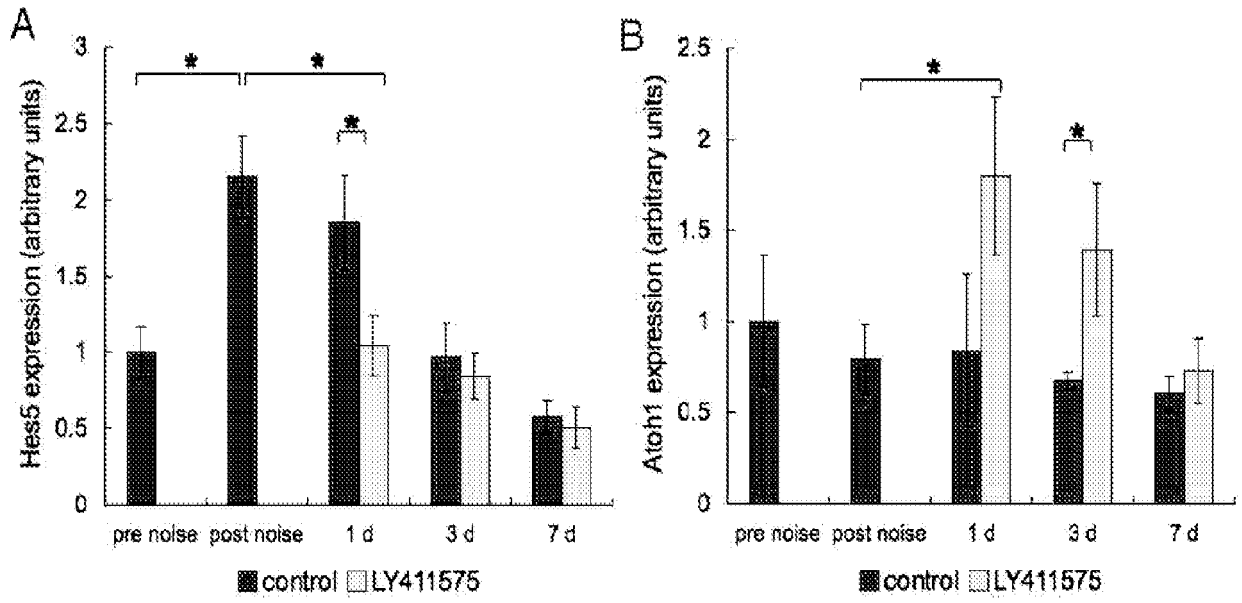
15. The method of any of the preceding claims, wherein the mammal is a human.



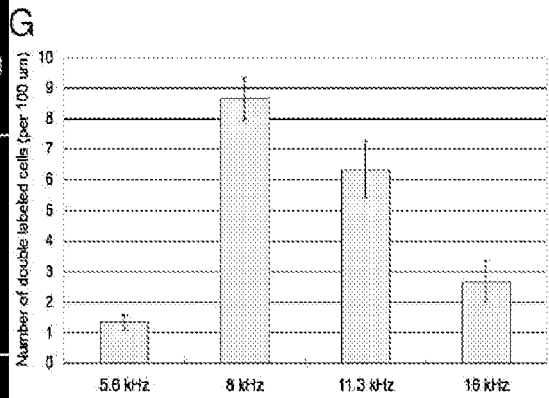
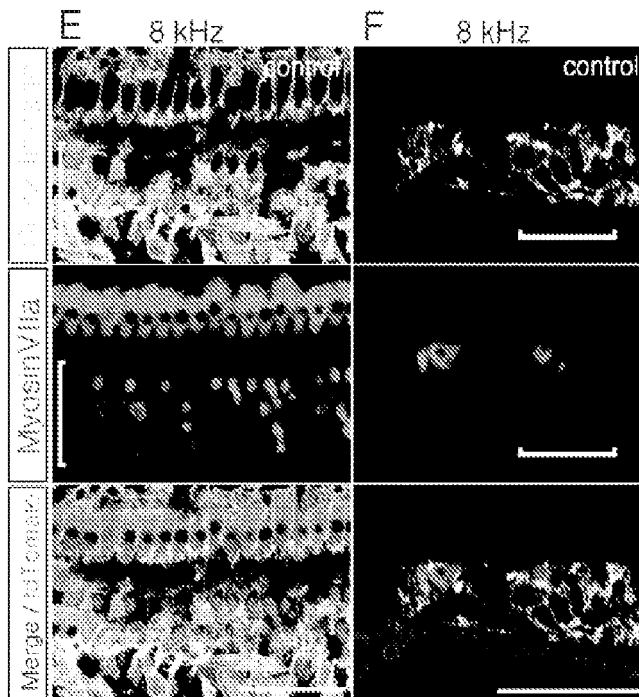
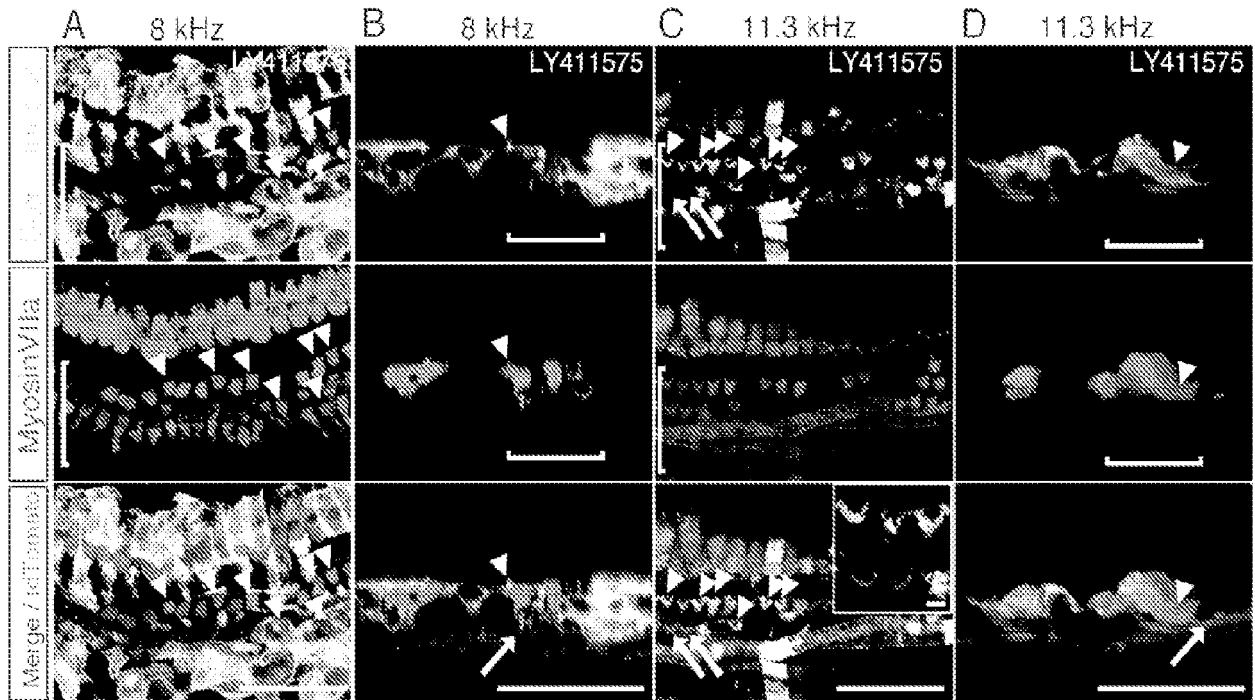
FIGS. 1A-D



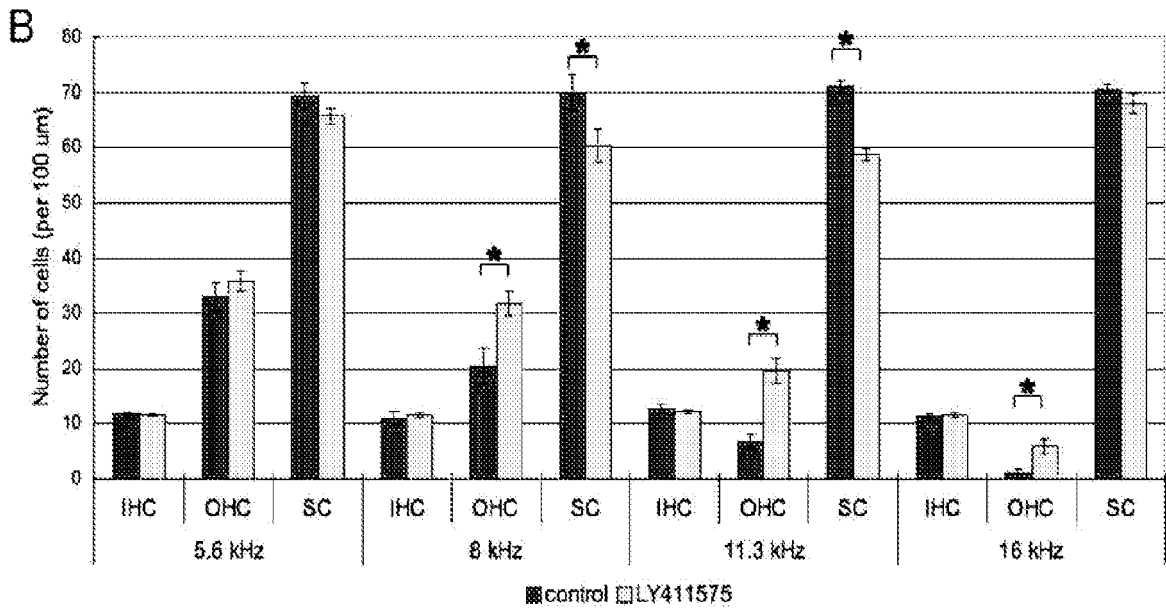
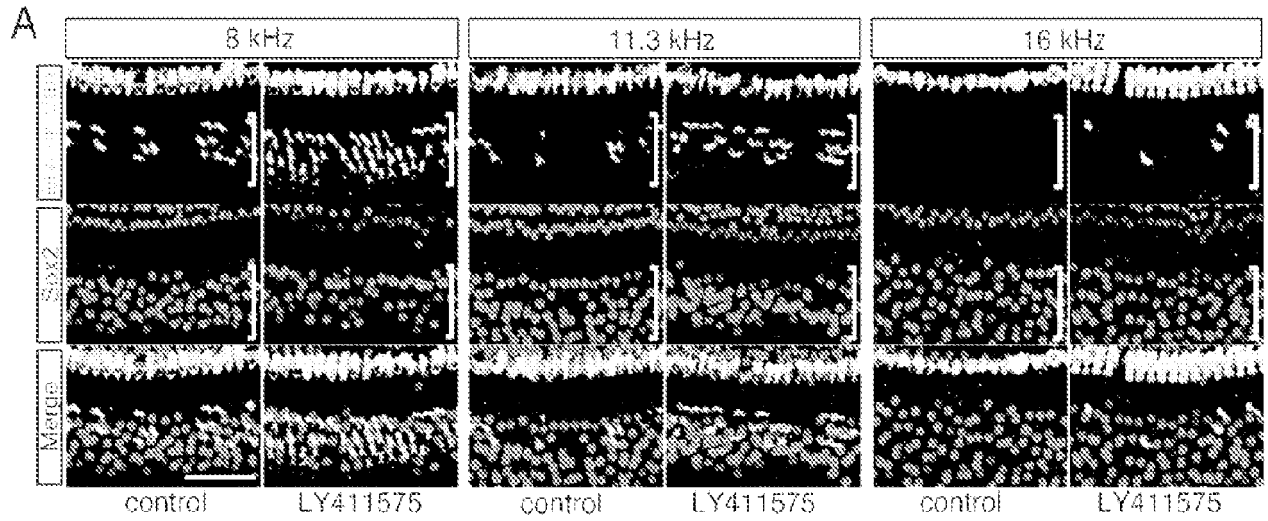
FIGS. 2A-D



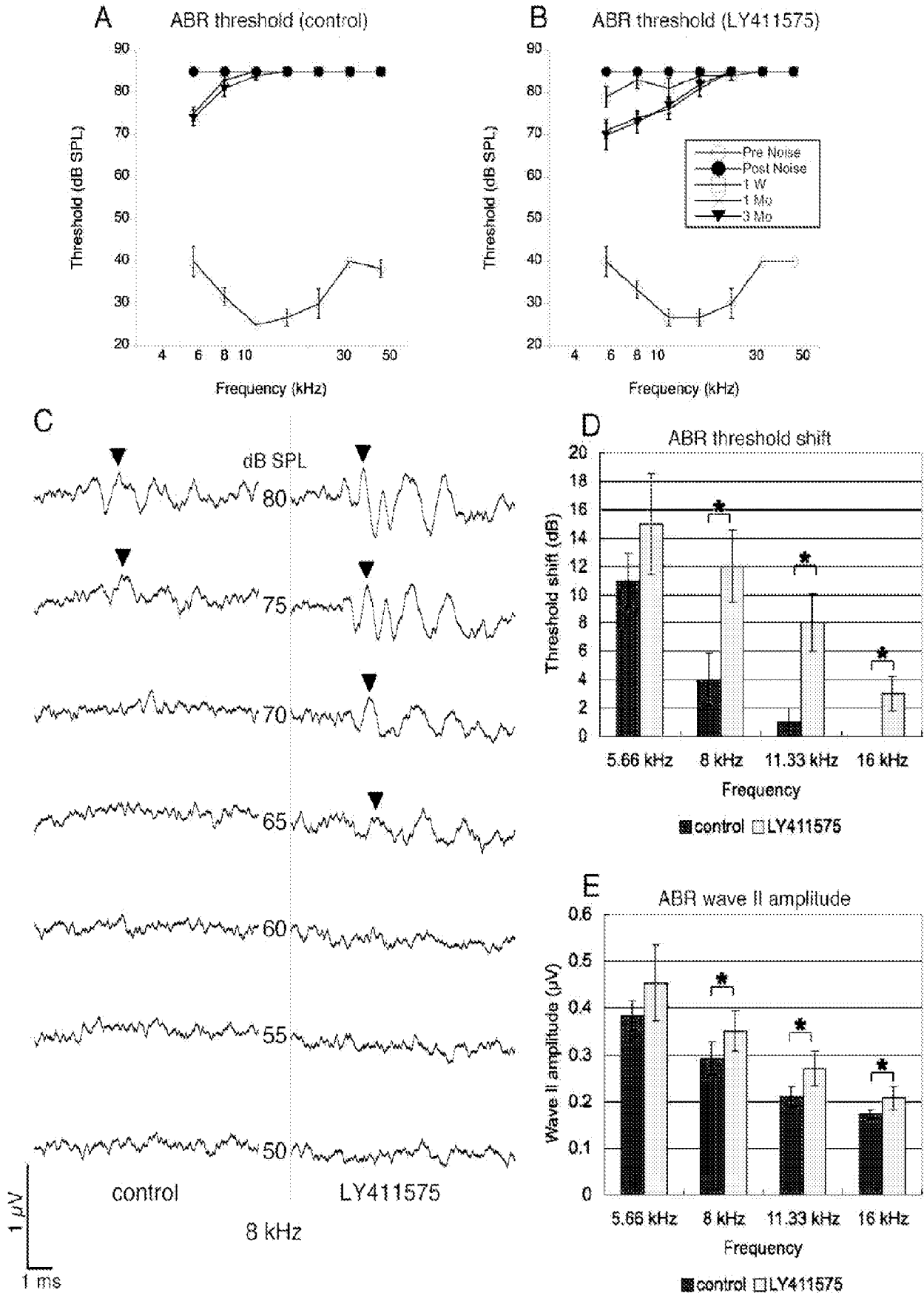
FIGs. 3A-B



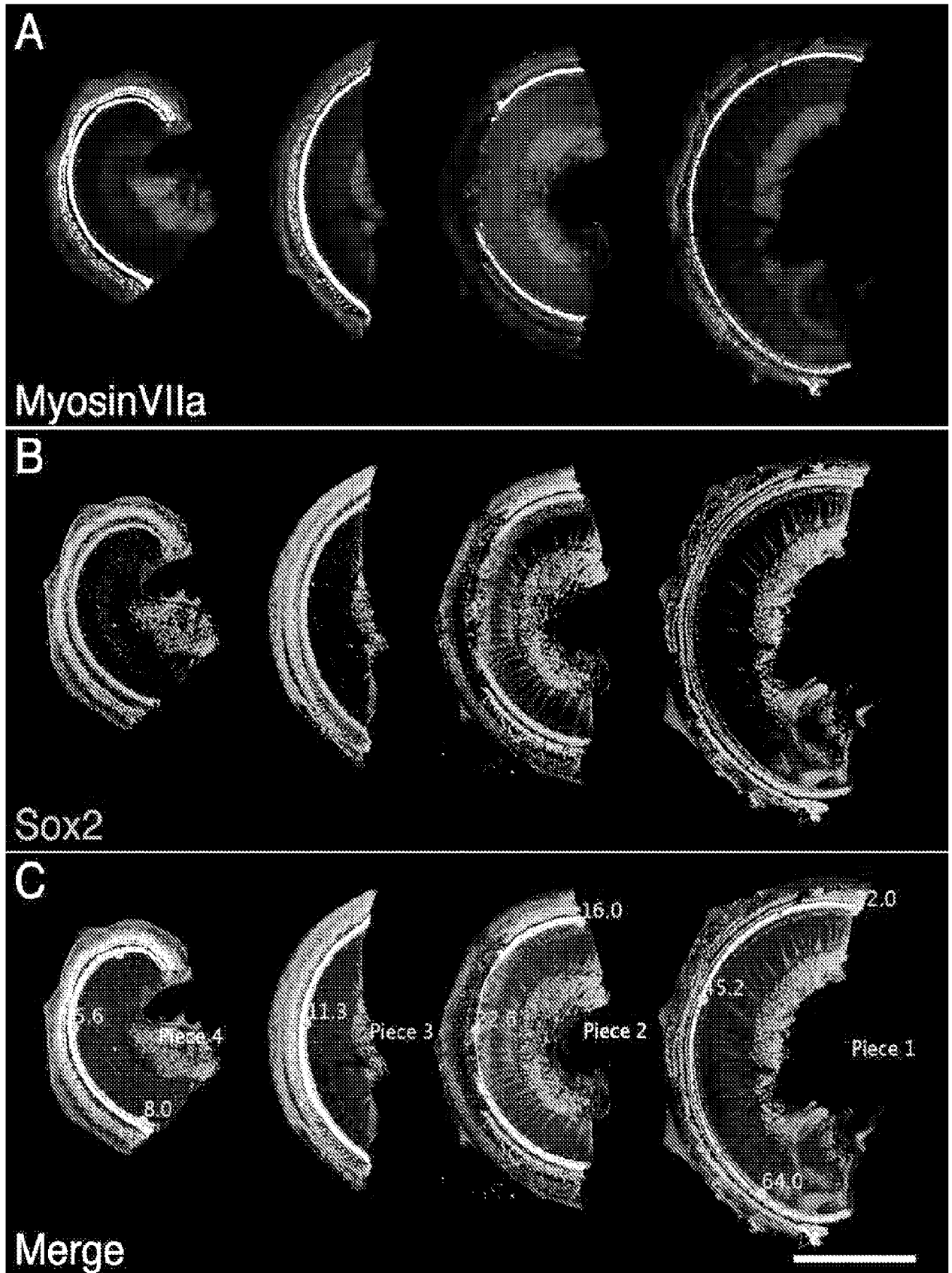
FIGs. 4A-G



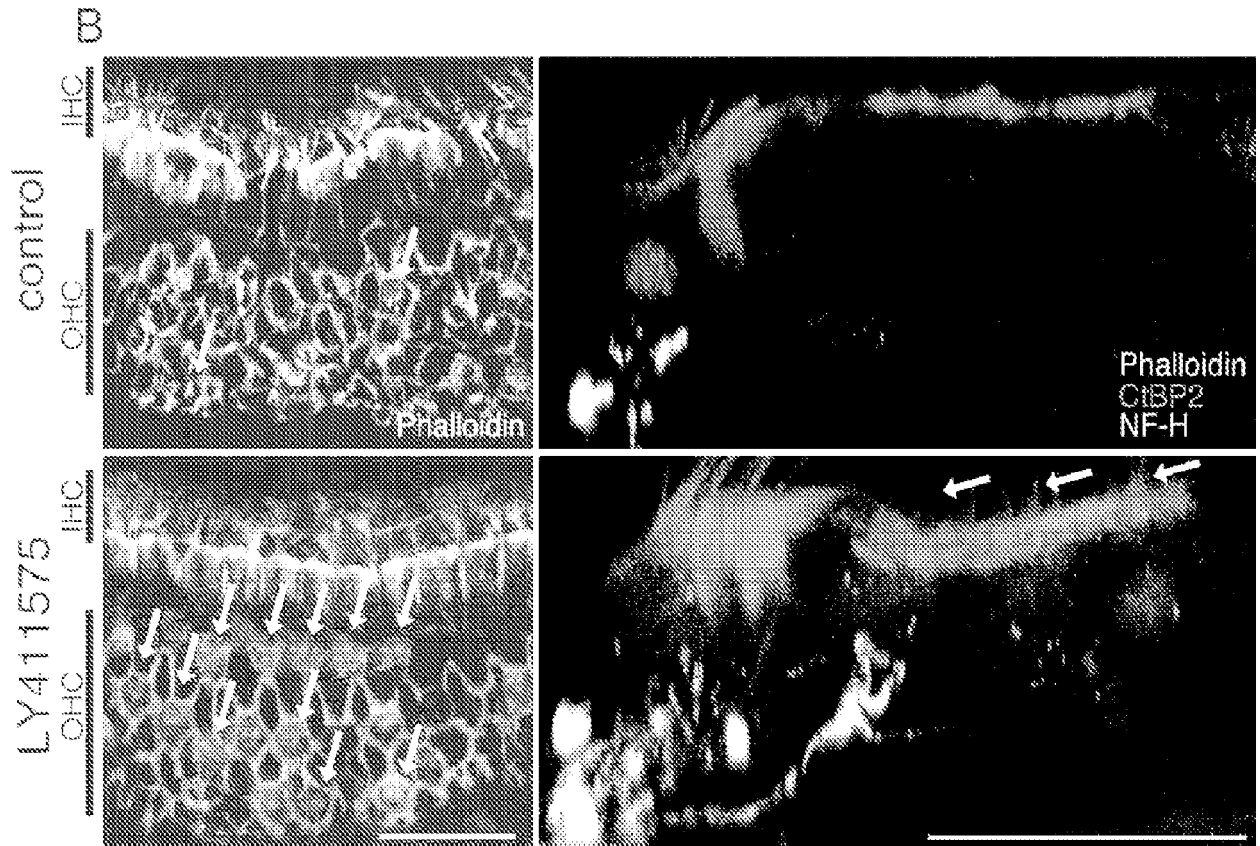
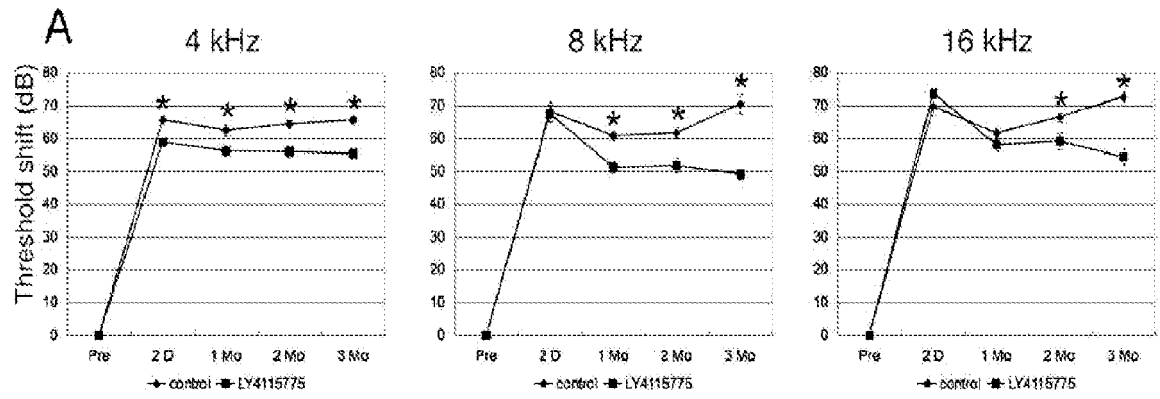
FIGs. 5A-B



FIGS. 6A-E



FIGS. 7A-C



FIGS. 8A-B

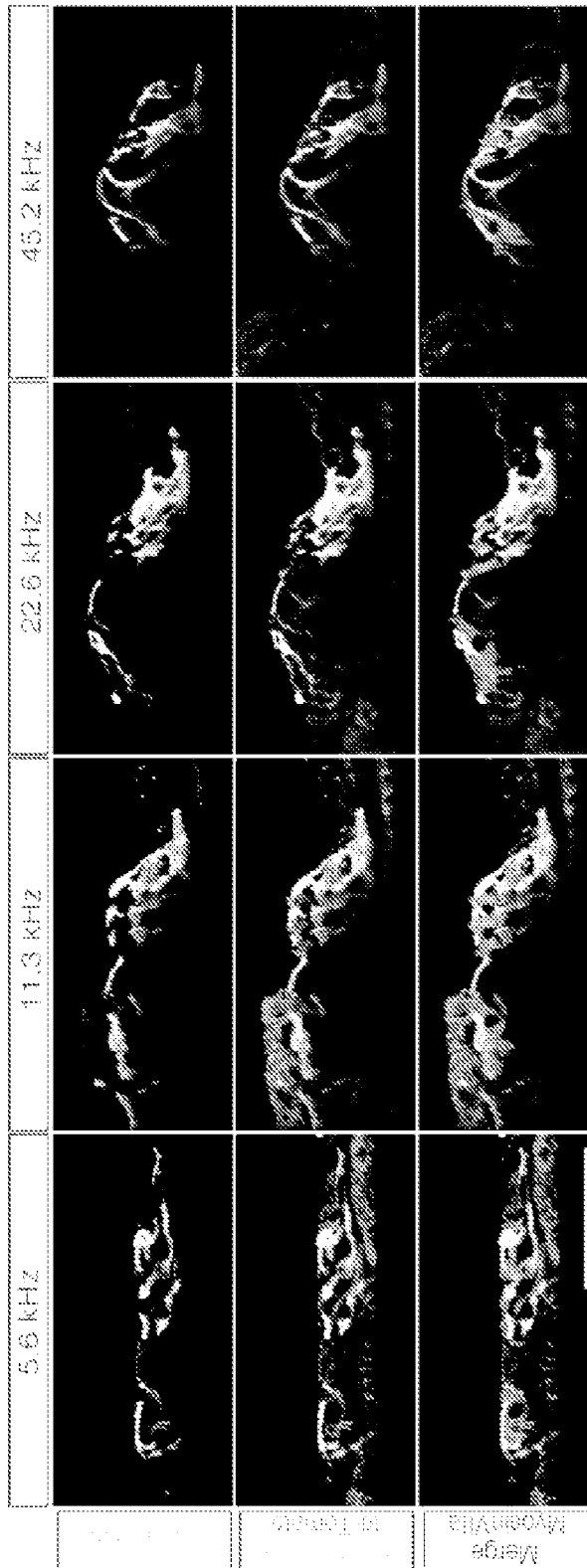


FIG. 9

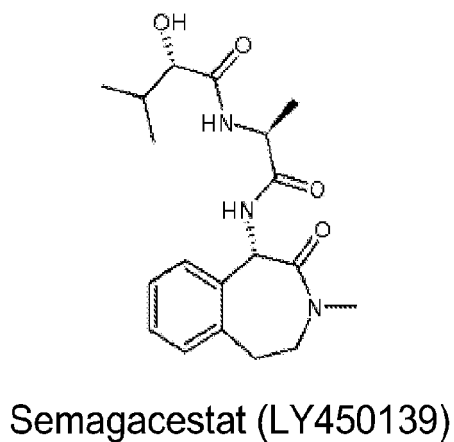
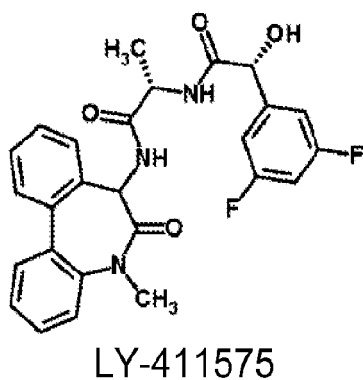
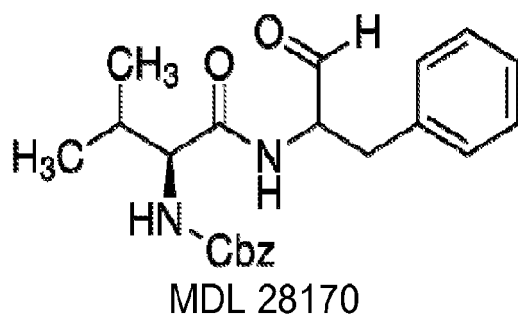
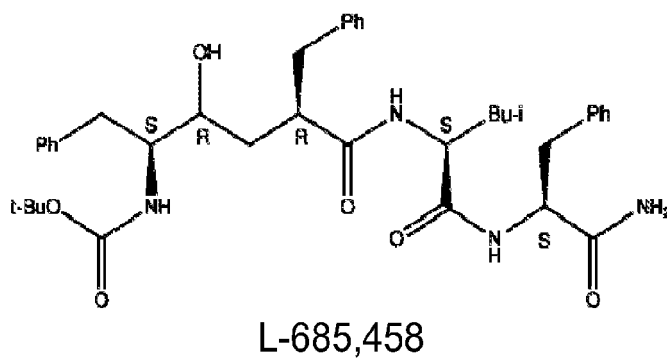
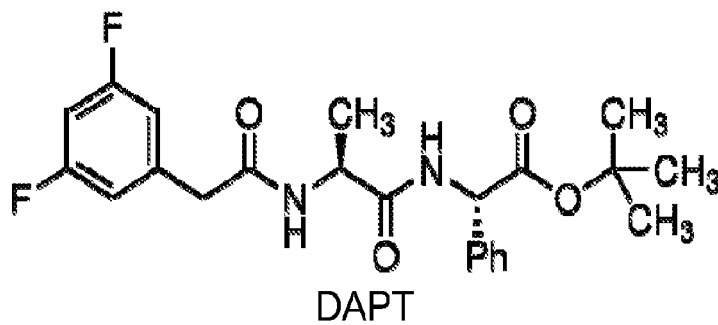
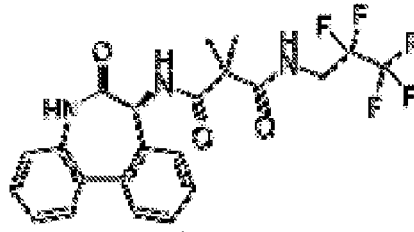
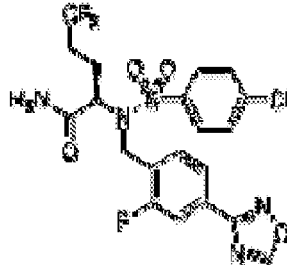


FIG. 10A

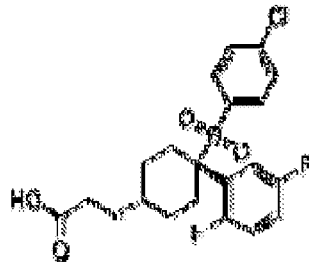
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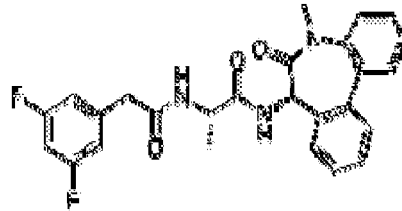
RO4929097



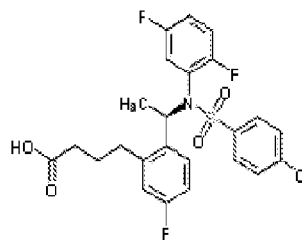
BMS-708163 (Avagacestat)



MK-0752



YO-01027



BMS 299897

FIG. 10B

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/058446

## A. CLASSIFICATION OF SUBJECT MATTER

*A61K 9/22 (2006.01)*  
*A61K 31/00 (2006.01)*  
*A61K 31/55 (2006.01)*  
*A61K 47/30 (2006.01)*  
*A61P 27/16 (2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 31/00, 31/55, 9/22, 47/30, A61P 27/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSearch (RUPTO internal), DWPI, RUPAT, Espacenet, PubMed, ScienceDirect, SpringerLink

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2009/0124568 A1 (MASSACHUSETTS EYE & EAR INFIRMARY) 14.05.2009, pp. 8-9, paragraphs [0075], [0077], [0080], [0081], p. 10-11, paragraphs [0086], [0091], [0095], [0096], claim 1	1-5, 8-10, 13-15 6, 7, 11, 12
Y	US 2009/0297533 A1 (OTONOMY, INC. et al.) 03.12.2009, abstract, p. 2, paragraph [0012], claim 1	6, 7
Y	LIN Vincent et al. Inhibition of notch activity promotes non-mitotic regeneration of hair cells in the adult mouse utricles. J Neurosci, 2011, 31(43), pp. 15329-15339, especially, p.15329, abstract	11, 12
Y	WOLFE Michael S. $\gamma$ -Secretase Inhibition and Modulation for Alzheimer's Disease. Curr Alzheimer Res., 2008, 5(2), pp. 158-164. Retrieved from Internet: <a href="http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2675939/pdf/nihms-107283.pdf">http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2675939/pdf/nihms-107283.pdf</a> , pp.1-21	11, 12

 Further documents are listed in the continuation of Box C. See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

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## INTERNATIONAL SEARCH REPORT

International application No.

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAMON Jeremy B. et al. Preclinical analysis of the gamma-secretase inhibitor PF-03084014 in combination with glucocorticoids in T-cell acute lymphoblastic leukemia. <i>Molecular Cancer Therapeutics</i> , 2012 April 13, Vol. 11, No. 7, pp. 1565-1575, doi:10.1158/1535-7163.MCT-11-0938, abstract	11, 12
Y	BASI Guriqbal S. et al. Amyloid precursor protein selective gamma-secretase inhibitors for treatment of Alzheimer's disease. <i>Alzheimer's Research &amp; Therapy</i> , 2010, 2:36. Retrieved from Internet: <a href="http://alzres.co/content/2/6/36">http://alzres.co/content/2/6/36</a> , pp.1-21, especially, p. 2, col. 2, paragraph 3, p. 5, col. 1, last paragraph, p. 13, col. 2, paragraph 3	11, 12
Y	US 2011/0033480 A1 (PARTHA SARKAR et al.) 10.02.2011, paragraph [0042]	11, 12
Y	MASUDA Shigeo et al. Dual antitumor mechanisms of notch signaling inhibitor in a T-cell acute lymphoblastic leukemia xenograft model. <i>Cancer Sci</i> , 2009, vol. 100, no. 12, pp. 2444-2450, especially, p. 2444, col. 2, paragraph 2	11, 12
Y	HUYNH C. et al. The novel gamma secretase inhibitor RO4929097 reduces the tumor initiating potential of melanoma. <i>PLoS One</i> , 2011, 6(9), e25264, 10.doi:10.1371/journal.pone.0025264, abstract	11, 12