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How Gata3 Affects Neuronal Survival in the Inner Ear

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Abstract

Broadly speaking, *Gata3* is a transcription factor involved in neurosensory specification and hair cell differentiation. Previous studies have shown that *Gata3* null mutants do not develop neurons, so *Gata3* appears to be necessary for neuronal survival. To better understand the role of *Gata3* in spiral ganglion neurons of the inner ear, a *Neurod1-cre* model was used to conditionally knock out *Gata3* at E9-E9.5, the timepoint when neuroblasts first begin to delaminate from the otic placode. The mutants used in this study were Nd1-cre: *Gata3* f/f mutants. *In situ* was performed to determine gene expression within the cochlea, and immunohistochemistry was performed to label neurons as well as cell death within the cochlea. When *Gata3* was conditionally knocked out and reduced in its expression, fewer neurons appear to have formed compared to the mutant and the neurons that do form lack normal projection patterns. Increased Activated Caspase3 labelling was found in the mutant compared to the control, meaning that there was increased cell death in the mutant. The Activated Caspase3 labelling follows the general pathway of the neurons, leading to the belief that neurons were the cells that were dying when *Gata3* was conditionally knocked out. Based on this, *Gata3* does appear to be necessary for SGN survival within the inner ear.

Introduction

Anatomy and Physiology of the Ear

The process of hearing is accomplished through the interaction of several physiological structures within the ear, which is separated into three parts: the outer, middle, and inner ear. Sound waves first interact with the outer ear, which has a funnel shape that allows for the optimal collection of sound. Sound waves then travel to the middle ear and contact the tympanic membrane, also known as the eardrum. Three structures in the middle ear known as the ossicles, the malleus, incus, and stapes, vibrate in that order before tapping on the oval window. The oval window vibrates, sending the sound waves to the inner ear, which consists of the cochlea, vestibule, and semicircular canals. The vibrations cause endolymphatic fluid within the cochlea to move, and this movement disturbs the basilar and tectorial membranes. The movement of the membranes bend the hair cells of the inner ear, which open ion channels and allow the hair cells to be depolarized (National Institute on Deafness and Other Communication Disorders, 2020). Hair cells sit on the basilar membrane within the organ of Corti and are divided into two categories: outer hair cells (OHC), which consist of three rows of hair cells, and a single row of inner hair cells (IHC). The differences are not only physical, but functional as well, with IHC responsible for the act of hearing and transducing auditory information, and OHC responsible for modifying sound. Once the hair cells are depolarized, neurotransmitters release onto the afferent neurons of the cochlea, also known as spiral ganglion neurons (SGNs). The afferent cochlear neurons comprise the cochlear branch of the eighth nerve. Before exiting the ear, they coalesce with afferent neurons from the vestibular system to form the vestibulocochlear nerve. This nerve transmits auditory information to the cochlear nucleus located in the hindbrain. Auditory information will continue to be processed within the central nervous system until finally ending at the auditory receiving centers located in the temporal lobe to be understood.

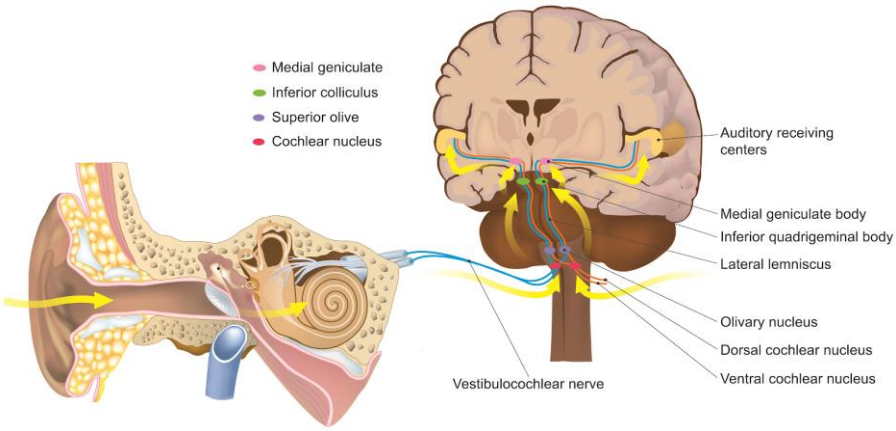

Figure 1. A diagram visualizing how sound travels from the ear to the brain. Sound waves are represented by the yellow arrow. (https://teachmeanatomy.info/neuroanatomy/pathways/auditory-pathway/)

Anatomy of Inner Ear Neurons Spiral ganglion neurons can be divided into two broad classes that differ in their physiological characteristics and in the hair cell types with which they interact. Type I spiral ganglion neurons innervate IHC and Type II neurons innervate OHC. These types of neurons differ slightly in their composition, with Type I neurons being larger, thicker, and containing myelinated processes, and Type II neurons being smaller, thinner, and having unmyelinated processes. The cell bodies of the neurons are located between the hair cells and the eight cranial nerve, with the projections going out to the hair cells known as peripheral projections, and the ones leading to the nerve called central projections. The central projections reach out

towards the hindbrain, and then they split to innervate the dorsal and ventral divisions of the cochlear nucleus (Appler and Goodrich, 2011).

Ear Development

Development of the inner ear in *Mus musculus* begins with formation of the otic placode, which is thickened ectoderm, that invaginates upon itself to form an otocyst. Bone morphogenic protein 4 (BMP4) and Fringe (*Fng*) are actively expressed throughout development and regulate growth and maturation of sensory organs within the inner ear. The semicircular canals are first able to be recognized at around embryonic day 12 (E12) (Morsli, et al., 1998). At E13 the semicircular canals are well formed, and the cochlea begins to expand and coil. The sensory organs reach their mature shape around E17.

Neuronal Development

Prior to the inner ear developing its structure, around E9, neuroblasts separate from the otic placode and form the cochlear-vestibular ganglion. As described in the review from Coate and Kelly, 2013, neuronal development first begins with the delamination of cells within the otic vesicle from the otic epithelium. The otocyst is divided into anterior and posterior regions, with the anterior otocyst holding the neural and sensory region, and the posterior otocyst holding the non-sensory region. Neuroblast delamination continues up until E12.5. From E12-E15.5, SGNs travel alongside the elongating cochlear duct away from the vestibule, eventually ending in the cochlear apex. The SGNs align themselves with supporting cell progenitors and hair cell progenitors at this time, although it is unclear how they distinguish from the other cell types within the otocyst. As development progress, central and peripheral projections extend from the neuronal cell bodies. The first central projections reach the hindbrain around E11.5 (Appler and Goodrich, 2011), while peripheral projections begin to form at E12.5. Peripheral axons are initially highly branched before being "pruned." The peripheral axons form radial bundles and then innervate hair cells within the organ of Corti.

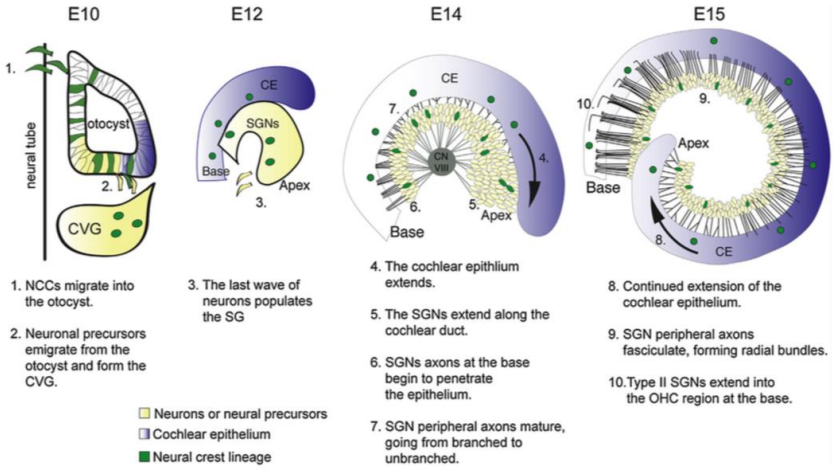

Figure 2. The development of neurons from E10-E15. (Kelly and Coates, 2013)

Genes Involved in Neuronal Development

Neuronal development occurs in a subset of cells within the proneurosensory domain of the inner ear. This domain contains cells that express proneurosensory genes, such as Sox2, Eya1, and Six1. In cells that will differentiate into neurons, the transcription factors Neurogenin1 (Ngn1) and Neuronal Differentiation 1 (Neurod1) are upregulated to specify a neuronal fate. *Ngn1* is the first gene to be expressed that marks cells' fate as a neuronal cell, and loss of this gene results in a loss of inner ear neurons (Appler et al., 2013). *Neurod1* is expressed as development progresses and guides cells through the mechanisms necessary to differentiate as neurons. The zinc-finger

transcription factor *Gata3* is also involved in spiral ganglion neuron differentiation. Gata3 expression first begins in the otic placode around E8 and is in developing spiral ganglion neurons as early as E10.5. (Duncan, et al. 2011). Broadly speaking, *Gata3* is involved in neurosensory specification and hair cell differentiation (Duncan and Fritzsch, 2013). *Gata3* has also been found to be involved in neuronal guidance (Appler et al., 2013); however, *Gata3* null mutants do not develop neurons, so it appears to be necessary for neuronal development (Duncan, et al. 2011).

Cre-*loxP* System

The mice used for these experiments were bred using the Cre-LoxP system, which allows for a gene to be deleted at a specific time point in a specific location of tissue. In this system, a bacteriophage is used to flank the gene to be excised. A male that has one floxed allele and has the Cre transgene is bred with a female homozygous for floxed sequences to produce a Cre:flox/flox mutant. As the gene in question is translated, so is Cre, which after being translated goes back into the nucleus and excises one LoxP site, removing critical exons for functionality, rendering the gene inactive.

Previous Studies/Implications

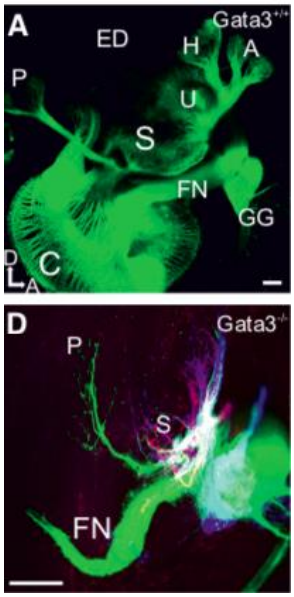

Figure 3. Lipophilic dye was inserted in rhombomere 4 at E16.5 to label the facial nerve and vestibulocochlear efferents and afferents to the ear. (Duncan et al. 2011)

Previous studies examining the role of *Gata3* in inner ear development showed that it is required for formation of hair cells in the organ of Corti and for spiral ganglion neuron differentiation (Duncan and Fritzsch, 2013). It is notable that in Figure 3, from Duncan et. al. 2011, the *Gata3* null mutant in panel D does not have any neurons. In Figure 4, a *Gata3* conditional knockout, the cochlear nerves were ablet to target the cochlear nucleus, however, when they reached the cochlear nucleus the fibers become disoriented and do not target the proper cell types. It is also unclear if the number of neurons is the same in the control (panel A) and in the mutant (panel C). Panels B and D map out the projections of a single neuron, and in the mutant, there are many more aberrant projections, which leads to the belief that there could be fewer neurons, but it looks dense due to the outgrowth of projections. In another study done, *Gata3* was shown to be necessary for SGN survival, and was involved mostly in peripheral projection growth (Appler et al., 2013). The model for our study differs in that the timing of Cre protein translation is slightly earlier at E9.5 and eliminates all neuroblasts at once.

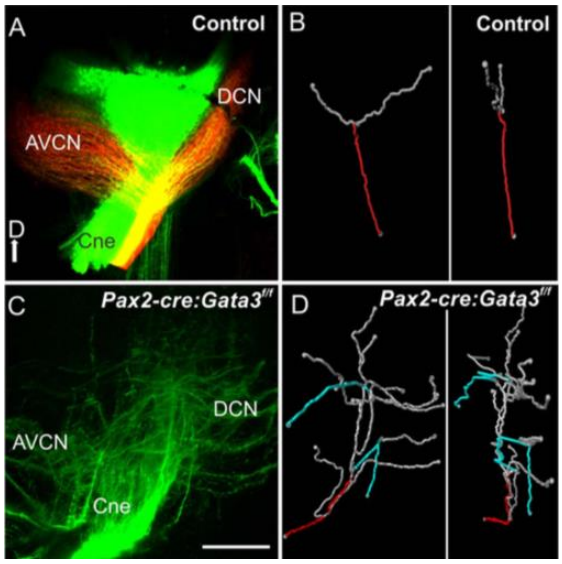

Figure 4. Lipophilic dye was placed into the cochlea of 18.5 mice, and Amira software was used to trace individual axons. (Duncan and Fritzsch, 2013)

My Hypothesis

My project is examining whether *Gata3* is necessary for spiral ganglion neuron survival. Based on previous studies described above, I hypothesize that *Gata3* is necessary for SGN survival, and conditional deletion of *Gata3* using our genetic mouse model will lead to SGN death.

Materials and Methods

Mice and L-DOPA Administration

Nd1-cre: *Gata3* f/f mice were produced by breeding Neurod1-cre: *Gata3* f/+ males with *Gata3* f/f females. All experiments were performed at E14.5.

The *Neurod1-cre* used in this study conditionally deletes *Gata3* at E9-9.5 and is lethal at E11.5 due to noradrenaline deficiency. To extend the lifespan to E14.5, L-DOPA was administered in the drinking water of the pregnant dam. At the noon of E6.5, L-DOPA and ascorbic acid powders were added to the drinking water of females. To make 100 mL of this solution, 2 mg of L-DOPA and 12.5 µL of 2% ascorbic acid was added to 100 mL of water.

Embryos were collected at E14.5. All animal studies were conducted with approval by the Institutional Animal Care and Use Committees at Western Michigan University (#20-11-01).

PCR/Genotyping

The primer sequences used for genotyping were CCT GTT TTG CAC GTT CAC CG ATG CTT CTG TCC GTT TGC CG CTA GGC CAC AGA ATT GAA AGA TCT GTA GGT GGA AAT TCT AGC ATC ATC C for Cre and both TCA GGG CAC TAA GGG TTG TTA ACT T GAA TTC CAT CCA TGA GAC ACA CAA and GAT TCA GTC TCC CTC CTT CTT C GTT CAC ACA CTC CCT GCC TTC TG for *Gata3 flox*.

Imaging

Imaging was done at a Nikon C2 confocal microscope. Images were compiled using Image J, and edited using CorelDRAW.

Immunohistochemistry

Blocking was performed in 5% donkey serum, and 0.5 g BSA in PBS at room temperature on a rocker. Samples were rinsed in PBS with 0.05% Tween-20 five times for five minutes and then placed in blocking solution for 20 minutes. Primary antibodies were diluted in PBS and included a neuronal marker, NF200 (1:200; Invitrogen), and a marker for apoptotic cells, Activated Caspase3 (1:200; Millipore). The samples were then incubated two nights at 4ºC. Samples were then rinsed four times with PBS for thirty minutes each. Secondary antibodies (Anti-Rabbit 647, Anti-Chick 555, Hoescht 405) were added at a 1:1000, 1:1000, 1:2000 dilution respectively in PBS overnight at 4ºC on a rocker. Samples were then rinsed 3 times thirty minutes in PBS and then mounted on a glass slide with glycerol and imaged using a Nikon C2 confocal microscope.

Average Immunofluorescence Quantification

Average immunofluorescence was quantified using a modification of the protocol. described in Duncan et al., 2019. A compiled Z-series image of only Activated Caspase3 labeling was created for both the control sample and the mutant sample. These images were analyzed using the 'histogram function' on ImageJ software, which automatically calculates the mean intensity of the

TIFF image. Corresponding average immunofluorescence values were then plotted so that relative fluorescence between control and mutant images could be compared.

In Situ

As described in Duncan and Fritzsch, 2013, control and conditional knock-out mice were fixed in 4% PFA and dissected in 0.4% PFA RNAse-free conditions. From this point forward, a control and conditional knock-out were always run in the same vial (one right ear and one left ear) to expose mutant and control to the same experimental conditions throughout. Ears were defatted in 100% methanol and rehydrated through a graded methanol series, digested with proteinase K (Ambion), and then hybridized to a specific riboprobe overnight at 60ºC. Unbound probe was washed off and the tissue was incubated overnight with anti-digoxigenin antibody (Roche) conjugated with alkaline phosphatase at room temperature. The probe was detected using BM Purple Ap Substrate (Roche). The tissue was mounted in glycerol and imaged using a Nikon E600 microscope. Care was taken to ensure microscope settings were identical for paired samples and images.

Results

An *in situ hybridization* experiment was performed to determine whether *Gata3* expression was truly eliminated out when using the *Neurod1-cre* line. Figure 5, Panel A depicts the control sample at E14.5. BM Purple expression is most highly concentrated in the base, and the presence of BM Purple indicates *Gata3 mRNA* expression. Compared to the control, the mutant sample in Panel B has decreased BM Purple labelling, and therefore has decreased *Gata3* mRNA expression.

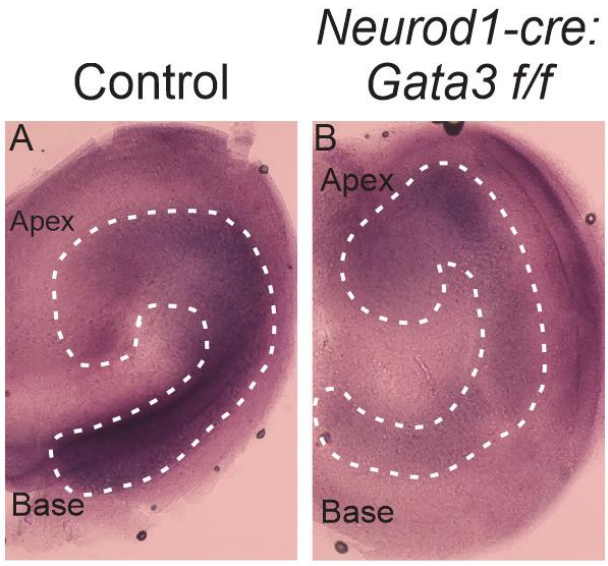

Figure 3. This figure shows the results of an in situ done at E14.5 within the cochlea on a control ear in panel A and a mutant ear in panel B. The mutant's genotype is Neurod1-Cre Gata3 f/f, as described above. The region within the dotted white lines hold the cell bodies.

Immunohistochemistry was performed on a mutant and control ear as well, which can be seen in Figure 6. The red labelling is from Neurofilament 200, which labels neurons, and the blue labelling is from Activated Caspase3, which labels dying cells. In Figure 6 Panel A, neurons appear normal, and branch outwards in a characteristic spiral pattern. Panel A' does show some Activated Caspase3 labelling, however, this is relatively normal for this developmental time point. In the mutant image, Figure 6 Panel B, neurons have an abnormal phenotype compared to the control. They appear to be significantly decreased in their numbers, and they lack the branching seen in the control image. B' shows increased Activated Caspase3 labelling compared to the control, and

the Activated Caspase3 labelling appears to be following the general area of neuron growth. Figure 6 quantifies the difference in Activated Caspase3 labelling between the control and mutant in a graph format. The results seem significant, but replicates are needed to confirm these findings.

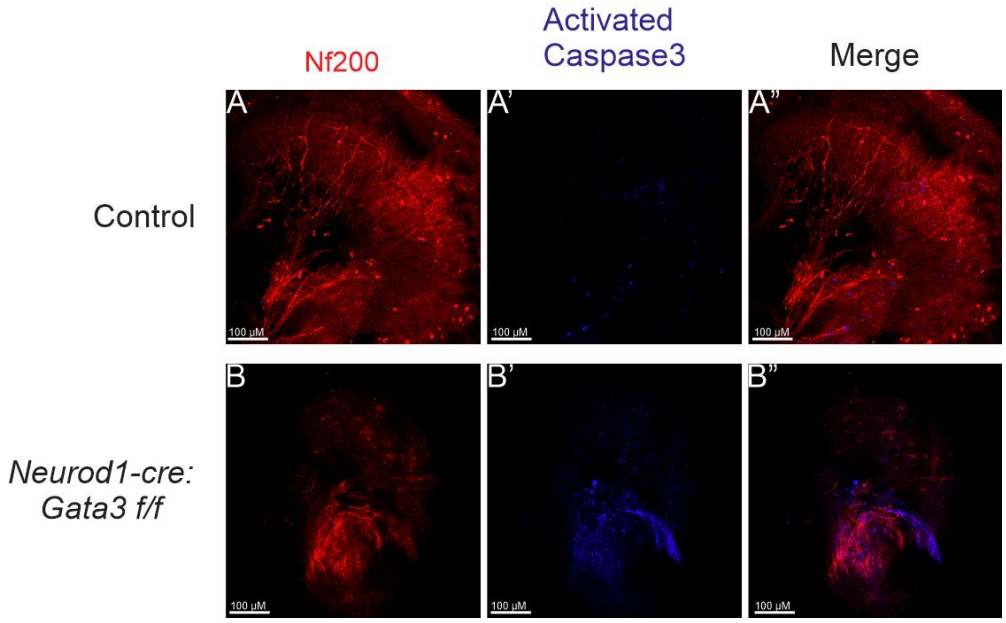

Figure 4. These images are from an immunohistochemistry experiment done on E14.5 ears, specifically looking at the cochlea of the inner ear. A-A" are from the control sample, and B-B" are from the mutant sample. Panels A and B show neurons stained in red with NF200, A' and B' show cell death labelled in blue with Activated Caspase3, and A" and B" combine panels A+A' and B+B' respectively.

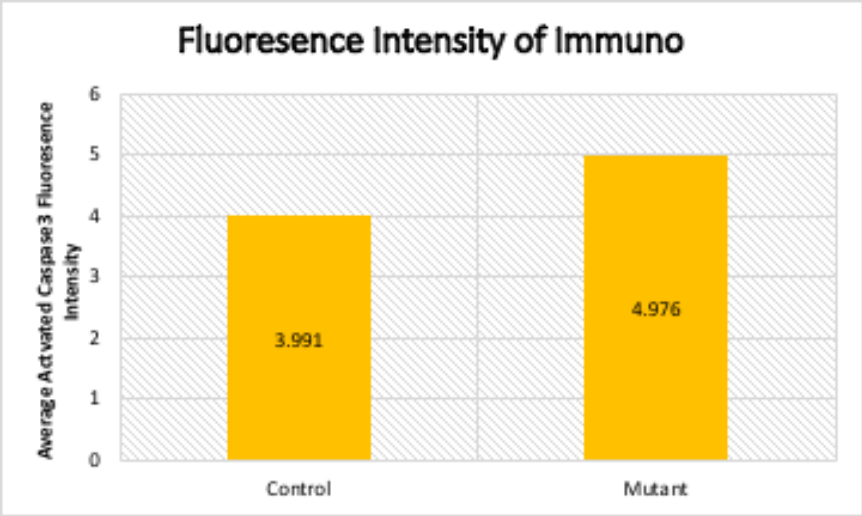

Figure 5. Quantification of fluorescent intensity of Activated Caspase3 in control and mutant samples following immunohistochemistry. Mutant samples have a higher average fluorescence, indicating a greater

Discussion

Based on the results of the *in situ hybridization* experiment, *Gata3* expression is reduced using the *Neurod1-cre* line; however, it is unclear if *Gata3* has been completely knocked out of spiral ganglion neurons. There is still BM Purple expression in the mutant image, meaning that *Gata3* is still being expressed. One explanation for this is that there could be *Gata3* expression in the glial cells rather than the neurons themselves. Regardless, *Gata3* expression is still markedly reduced in the mutant compared to the control. In the future, qPCR data should be collected to quantitatively determine *Gata3* expression within the mutant and control ears. Due to time constraints, this could not be done at the time of this study. The immunohistochemistry experiment showed increased Activated Caspase3 labelling in the mutant, meaning that there was increased cell death in the mutant. The increased labelling, combined with the neuronal pattern the labelling follows, as well as the apparent decrease in neurons leads to the belief that the cells that died were actually neurons. Based on this, *Gata3* does appear to be necessary for SGN survival within the inner ear. Further experimentation should be done to provide replicates in immunohistochemistry to confirm these findings, and qPCR data should be used in the future to quantify the reduction in *Gata3*. The implications of these findings could be applied in gene therapy for patients with hearing loss due to neuron loss. The transplantation of pluripotent stem cells has been successful in several animal models, however, regaining the functionality of damaged neurons and hair cells has not yet been realized, and long-term survival also remains a problem in these models (Tang et al., 2020). Based on my findings, in the future, increased *Gata3* expression could potentially be used in conjunction with stem cell treatment to promote new neuron growth and ensure neuron survival.

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References

Appler, J. M., & Goodrich, L. V. (2011). Connecting the ear to the brain: Molecular mechanisms of auditory circuit assembly. *Progress in Neurobiology, 93*(4), 488-508. doi:10.1016/j.pneurobio.2011.01.004

Appler, J. M., Lu, C. C., Druckenbrod, N. R., Yu, W., Koundakjian, E. J., & Goodrich, L. V. (2013). Gata3 is a critical regulator of cochlear wiring. *Journal of Neuroscience, 33*(8), 3679-3691. doi:10.1523/jneurosci.4703-12.2013

Coate, T. M., & Kelley, M. W. (2013). Making connections in the inner ear: Recent insights into the development of spiral ganglion neurons and their connectivity with sensory hair cells. *Seminars in Cell & Developmental Biology, 24*(5), 460-469. doi:10.1016/j.semcdb.2013.04.003

Davies, K., & Sugano, Y. (2020, December 14). The auditory pathway. Retrieved April 27, 2021, from https://teachmeanatomy.info/neuroanatomy/pathways/auditory-pathway/

Duncan, J. S., & Fritzsch, B. (2013). Continued expression of gata3 is necessary for cochlear neurosensory development. *PLoS ONE, 8*(4). doi:10.1371/journal.pone.0062046

Duncan, J. S., Lim, K., Engel, J. D., & Fritzsch, B. (2011). Limited inner ear morphogenesis and neurosensory development are possible in the absence of gata3. *The International Journal of Developmental Biology, 55*(3), 297-303. doi:10.1387/ijdb.103178jd

How do we hear? (2020, December 14). Retrieved April 20, 2021, from https://www.nidcd.nih.gov/health/how-do-we-hear#:~:text=Sound%20waves%20enter%20the%20outer,malleus%2C%20incus%2C%20and%20stapes.

Lim, K., Lakshmanan, G., Crawford, S. E., Gu, Y., Grosveld, F., & Douglas Engel, J. (2000). Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. *Nature Genetics, 25*(2), 209-212. doi:10.1038/76080

Luo, X., Deng, M., Xie, X., Huang, L., Wang, H., Jiang, L., . . . Gan, L. (2013). GATA3 controls the specification of prosensory domain and neuronal survival in the MOUSE COCHLEA. *Human Molecular Genetics, 22*(18), 3609-3623. doi:10.1093/hmg/ddt212

Morsli, H., Choo, D., Ryan, A., Johnson, R., & Wu, D. K. (1998). Development of the mouse inner ear and origin of its sensory organs. *The Journal of Neuroscience, 18*(9), 3327-3335. doi:10.1523/jneurosci.18-09-03327.1998

Tang, P., Hashino, E., & Nelson, R. F. (2020). Progress in modeling and targeting inner ear disorders with pluripotent stem cells. *Stem Cell Reports, 14*(6), 996-1008. doi:10.1016/j.stemcr.2020.04.008