Developmental, Physiological, and Functional Neurobiology of the Inner Ear

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Andrew K. Groves

Department of Neuroscience, Baylor College of Medicine, Houston, TX, USA

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Chapter 1

Pou4f3^{DTR} Mice Enable Selective and Timed Ablation of Hair Cells in Postnatal Mice

Jennifer S. Stone, Edwin W. Rubel, and Mark E. Warchol

Abstract

Experimental studies of inner ear development and regeneration, as well as investigations of the influences of sensory input on CNS development, often require a rapid and nearly complete elimination of the hair cells of the inner ear at any postnatal age. Although these cells can be killed by noise trauma or by exposure to ototoxic drugs, both of these interventions are highly variable in their efficacy, resulting in considerable differences in sensory functions among individual animals that receive the same treatment. Furthermore, much current research of the auditory and vestibular systems is conducted using mice, and the ears of mice are relatively resistant to the effects of many ototoxins. In response to these concerns and others, the Rubel and Palmiter labs at the University of Washington developed a transgenic mouse line (called *Pou4f3*^{DTR}) in which the human form the diphtheria toxin receptor (also known as HB-EGF) is expressed under regulation of the *Pou4f3* promoter. Because *Pou4f3* is expressed by all hair cells (and relatively few other cells in the body), this mouse model permits the selective elimination of hair cells via 1–2 systemic injections of diphtheria toxin. This mouse line has been successfully used in studies of auditory CNS development and hair cell regeneration. This chapter provides an overview of this model, as well as detailed protocols for its use.

Key words Hair cells, Cell death, Mouse model, Deafness, Diphtheria toxin, Auditory system, Vestibular system, Regeneration

1 Historical Background

The sensory hair cells of the inner ear detect sound vibrations (in the cochlea) and head position and motion (in the vestibular organs) and convey this information to the brain via synapses upon the eighth cranial nerve. Injury or death of hair cells is relatively common in humans and can lead to permanent hearing loss, disequilibrium, and vertigo. The causes are varied and are likely to consist of a combination of genetic predisposition, along with a history of ototoxic drug exposure, noise exposure, or other forms of injury. Given the high prevalence of these conditions, it is of great interest to understand how hair cells die, how their death (and

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subsequent lack of neural input) affects other regions of the nervous system, and also how hair cells might be replaced or regenerated. Basic research on all of these issues relies on the use of animal models.

Early research on the function and pathology of the inner ear has employed a number of mammalian and non-mammalian models (e.g., [1, 2]). Insights into the operation of the cochlea were largely derived from studies of guinea pigs (which have a large and readily accessible cochlea), chinchillas (which also have an accessible cochlea and a hearing range similar to humans), and cats (which are advantageous for single unit physiology at all levels of the auditory system). Beginning in the 1990s, however, much of the research on the inner ear has involved the use of mice. Adoption of mice as a common animal model is attributable in part to the development of powerful tools for genetic sequencing and manipulation in mice, which has greatly enhanced our knowledge of the genetic and molecular basis of inner ear dysfunction. Mice are also easy to breed and maintain in laboratory environments. Apart from their genetic advantages, however, mice are not an optimal model for the study of hearing. The hearing range of mice (~4–60 kHz) is very different from that of humans (~0.02–20 kHz), which may be indicative of differences in the mechanics of the cochlea. In addition, quantifying the physiological function of the cochlea (e.g., via the recording of cochlear microphonics or the sound-evoked responses of single afferents) in mice is experimentally challenging. Similarly, mice are somewhat ill-suited for the study of vestibular function. Their vestibular sensory organs are small and relatively inaccessible, and their quadrupedal location makes it difficult to detect subtle changes in balance function. Mice also have small laterally positioned eyes, and their vestibulo-ocular reflexes (VORs) are difficult to quantify. Still, mice have emerged as very productive models in the studies of aging, ototoxicity, and acoustic trauma; the genetic homogeneity of inbred strains can minimize the degree of variability that is common for these types of insults.

The initial impetus to develop a transgenic mouse for time-sensitive, targeted ablation of hair cells was to better study the role of experience on shaping the development of structure and function of the brain. These types of studies can be traced back at least to the observations and writings of Aristotle. In the modern era, this includes the contributions of D. O. Hebb, visual system scientists like Austin Riesen, David Hubel and Torsten Wiesel, neuroembryologists like Victor Hamburger and behaviorists like Konrad Lorenz and Gilbert Gottlieb.

Building on the classic studies of auditory system embryology of Ramon y Cajal and Levi-Montalcini, and of visual system deprivation, the laboratory of one author (EWR) studied the role of synaptic activity on development of auditory pathways in birds and mammals for over four decades. One important observation made

by this group and others was that while damage to the inner ear prior to the onset of hearing had rapid and dramatic consequences on cochlear nucleus neuron survival, the response was age-dependent; the same manipulation a few days later resulted in no or minimal cell death. These studies provided further examples of critical periods wherein normal CNS development relies on synaptic inputs from the periphery (reviewed in [3–7]).

One of the challenges for these studies in the auditory system as well as other pathways has been how to remove or manipulate synaptic activity in areas of the brain in quantifiable ways without destroying other cellular components or creating other pathologies in inner ear or brain regions under investigation. In vivo approaches applied in the auditory field have included raising animals with ear plugs or in environments with abnormal acoustic experiences. While it is obvious that such manipulations change the *pattern* of activity, the unusually high levels of spontaneous activity of auditory neurons were likely not significantly altered under these conditions (e.g., [4, 8]). Another approach to silencing synaptic input to the brain included removal of the cochlea, as first shown by Kiang [9]. However, this manipulation did successfully eliminate excitatory synaptic activity at the level of the ventral cochlear nucleus, and it created pathologies in the inner ear, the nerve, and the cochlear nucleus. Investigators also pharmacologically blocked eighth nerve action potentials (e.g., [10–12]). While this approach was used in mature animals to great advantage, it is cumbersome, requires monitoring, and is difficult to validate in young animals.

Since the most common cause of hearing loss in humans is loss of hair cells, it makes sense to use a method that eliminates hair cells but does not damage other cochlear or CNS cells for altering synaptic input to the brain. Unfortunately, none existed. Therapeutic medicines that killed hair cells also damage other cochlear structures, and we do not know their direct effects on CNS neurons. Some genetic manipulations were becoming available (e.g., [13]) but usually limited to induction only in young animals. The obvious solution was to use genetics to remove or silence hair cells or auditory nerve axons in developing and mature animals.

Discovery of hair cell regeneration in birds [14, 15] brought attention to a new methodological problem. How could one distinguish the difference between a native (original) hair cell, a regenerated hair cell, and a hair cell that had been injured, had changed the expression of marker proteins, and had recovered? While tritiated thymidine or other cell cycle markers were immediately used to distinguish recently divided offspring [16, 17], it was quickly discovered that new hair cells could also arise by direct transdifferentiation (a non-mitotic conversion supporting cells in the sensory epithelia into hair cells [18, 19]) and that this method was the primary way in which hair cells were naturally regenerated in rodents [20–22]. While ototoxins and noise were available to

induce hair cell death, these methods had problems (discussed below) due to high rates of animal mortality, high lesion variability, off-target effects, or age restrictions.

Careful consideration revealed that the ideal mouse model for studying the role of synaptic activity on auditory brain development and hair cell regeneration should: (1) be inducible at any age; (2) be highly specific to hair cells (no off-target effects in key cell types in the inner ear or CNS); (3) work rapidly, in a matter of hours or a few days; (4) be reliably quantifiable and consistent across subjects or complete; (5) be non-invasively inducible; and (6) be effective in vitro and in vivo. Fortuitously, Richard Palmiter's group at the University of Washington had developed a method to selectively ablate hypothalamic neurons that control feeding behavior in mice [23, 24], as discussed in more detail in Subheading 3. The application of this method to create *Pou4f3*^{DTR} mice resulted in a highly useful model for time-controlled and selective ablation of hair cells in postnatal mice.

2 Traditional Methods for Inducing Hair Cell Death in Mice

The exact method used to create a hair cell lesion in mice depends on the goals of the study. If the objective is to identify the cellular mechanisms that underlie hair cell death caused by specific ototoxins (e.g., aminoglycoside antibiotics or the chemotherapy agent cisplatin), then the use of those specific drugs is mandated. On the other hand, studies focused on the consequences of inner ear injury (such as CNS plasticity, effects of sensory deprivation, or hair cell regeneration) will require creation of a hair cell lesion, but the actual method used to kill hair cells may not be critical. Instead, it may be more important that the lesion method is reliable and consistent, so that all experimental animals experience the same kind and extent of hair cell loss, or complete killing of all hair cells in the organ. Furthermore, it is often desirable that other cell types in the ear, such as organ of Corti supporting cells, peripheral and central neurons in auditory and vestibular pathways, or stria vascularis and dark cells, are not adversely affected by the method intended to kill hair cells.

2.1 Cochlear Hair Cells

The most common method for lesioning cochlear hair cells is to expose animals to intense sound. Before employing this technique, several species-specific parameters must be considered. First, the frequency range of hearing sensitivity varies greatly among mammals, so the sound frequency (or frequency band) to be used must be optimized for the specific animal species that is being studied. Also, the cellular effects of noise vary with the intensity and duration of exposure. In most strains of laboratory mice, moderately loud sounds (~90 dB SPL) will induce injury to afferent synapses

but will not impact hair cells or supporting cells [25, 26]. In contrast, exposure to higher sound levels (≥106 dB SPL) will kill cochlear hair cells, and intense sound (>116 dB SPL) can disrupt the integrity of the sensory epithelium [27]. Noise-induced hearing loss is common in humans, so identifying the mechanisms by which noise damages the cochlea is of great translational interest. However, the effects of noise on cochlear hair cells can be highly variable, such that the lesion induced by a particular noise regimen can vary from animal to animal. For this reason, unless noise exposure is a critical aspect of the rationale for a particular experiment, noise trauma is probably not the best option for ablating cochlear hair cells.

Another option for inducing cochlear injury involves treatment with ototoxic drugs. Most studies using drugs to kill hair cells in mice have used either aminoglycoside antibiotics or platinum-containing chemotherapeutic drugs that are widely used to treat tumors. Unfortunately, mice are often a suboptimal model for such studies. Killing cochlear hair cells with aminoglycosides requires delivery of multiple injections over 1–3 weeks, with doses that approach systemic toxicity [28, 29]. Aminoglycoside-induced death of cochlear hair cells is greatly enhanced when the antibiotic is administered in combination with a "loop" diuretic (e.g., furosemide, ethacrynic acid, bumetanide; [30]), and this approach has been used to create ototoxic lesions in mice [28, 31, 32]. The precise mechanism of the pharmacological interaction between loop diuretics and aminoglycosides is not fully understood, but it is likely that the diuretic permits increased transport of aminoglycosides across the stria vascularis, leading to higher concentrations of those drugs in the cochlear fluids. From an experimental standpoint, co-administration of loop diuretics and aminoglycosides is a reasonably reliable method for inducing hearing loss in mice. While aminoglycosides (with or without loop diuretics) mainly target outer hair cells and—at higher doses—inner hair cells, these treatments often cause damage or death of other cells in the organ of Corti, in the stria vascularis, and/or amongst spiral ganglion neurons. Also, the extent of damage to hair cells, and probably other cell types, varies dramatically as a function of age.

Cisplatin and other chemotherapeutic drugs have been used to lesion cochlear hair cells since the early 1970s (when those drugs were first developed). However, their use in mice presents similar challenges to those encountered with aminoglycosides. A clinically relevant protocol for cisplatin ototoxicity in mice has recently been developed [33], and it involves giving mice three 4-day courses of systemic injections of cisplatin, with each series separated by a 10-day drug-free "recovery" period. However, creating a cisplatin lesion with this protocol in mice requires 2–3 months and careful attention to detail. Finally, it should be noted that both high doses of cisplatin and intense noise can damage supporting cells [34–37],

stria vascularis [38, 39], and/or neurons (e.g., [40–42]) as well as hair cells.

A few transgenic mouse lines have been used to destroy hair cells in mice. Fujioka et al. [43] engineered *Pou4f3-Cre;Mos-iCsp3* mice in which treatment in vivo or in vitro with a drug called AP20187 results in mosaic, partial killing of the auditory hair cell population. This method should also work to achieve partial killing of vestibular hair cells. Additionally, mice generated by crossing *Atoh1-Cre*^{ER} mice to *Rosa26-stop-loxp-DTA* mice show near-complete killing of hair cells in both the cochlea [44] and vestibular organs [45] when tamoxifen is injected in the neonatal period. Unfortunately, this method does not work in adult mice because *Atoh1-Cre*^{ER} expression is lost in hair cells as mice mature.

2.2 Vestibular Hair Cells

The traditional method for killing vestibular hair cells in non-mammals and large rodents (guinea pigs) is to treat animals with a series of subcutaneous injections of aminoglycoside antibiotics. However, this approach is problematic in mice. In adult mice, kanamycin fails to induce any substantial hair cell loss in the utricle [31, 32] and, presumably, other vestibular organs. The high doses of aminoglycosides that are apparently required to kill vestibular hair cells in vivo are lethal to mice, and unlike in the cochlea, supplementation with diuretics such as furosemide does not enhance vestibular hair cell loss [31, 32]. Intralabyrinthine delivery of gentamicin creates near-complete lesions in adult mice [46], but this approach requires surgery.

There have been very few studies examining cisplatin as an inducer of vestibular hair cell damage in mice. Although small changes in a vestibular reflex and motor behaviors have been noted after cisplatin treatment [47], studies have found little or no loss of vestibular hair cells following cisplatin treatments at different doses and schedules [33, 47]. Furthermore, Fernandez et al. [33] detected no change in vestibular stimulus-evoked electrical potentials in the brain stem. There are other disadvantages to using cisplatin to kill hair cells including its lethal effects on supporting cells and neurons (*see* Subheading 2.1). Therefore, similar to aminoglycosides, cisplatin seems to pose technical limitations for studies requiring extensive and precise loss of hair cells.

Another toxin, 3,3′-Iminodipropionitrile (IDPN), has been employed to destroy vestibular hair cells in mice (e.g., [48, 49]). Although IDPN is easily administered to mice, it can cause pathological changes in different regions of the rodent body including the kidney, liver, and brain (e.g., [50–53]). In rats and mice, IDPN induces loss of hair cell-afferent nerve synapses in both vestibular and cochlear organs [54–56] and structural and molecular changes in the hair cell-calyx junction [56]. In addition, at high doses, IDPN can cause supporting cell death [57]. Most studies of IDPN ototoxicity have focused on the vestibular organs and

IDPN's effects on the cochlea are not well-characterized. The off-target effects of IDPN can make it challenging to isolate behavioral or physiological effects of hair cell loss and regeneration, which is important in many studies.

3 Selective Cell Ablation in Mice Expressing the Human Diphtheria Toxin Receptor

To minimize the off-target effects and extend the age range of treatment that accompany the more traditional methods for hair cell ablation, Edwin Rubel and Richard Palmiter engineered the *Pou4f3*^{DTR} transgenic mouse line [58–60]. In this line, the human diphtheria toxin receptor (hDTR) is expressed specifically on hair cells, thus allowing those cells to be selectively ablated upon administration of diphtheria toxin (DT also called DTx). Diphtheria toxin is a bacterial protein that kills cells by inhibiting protein synthesis [51, 61–63]. The DT molecule has two functional domains: DT-B, which is the receptor-binding and transactivating domain (which facilitates entry into the cell), and DT-A, which is the catalytic, toxic domain. DT binds to a receptor on the cell surface, which has been shown in some cells to be the precursor of heparin-binding epidermal growth factor like growth factor (HB-EGF) [64]. HB-EGF, which is expressed in many cell types, acts as a ligand and regulates a variety of cellular behaviors [65]. However, because HB-EGF can bind to and internalize DT, it is commonly referred to as the "DT receptor" (DTR). Once bound to HB-EGF, DT becomes incorporated into clathrin-coated pits and then endosomes. In the acidic environment of the endosome, the toxin becomes translocated to the cytosol, where it enzymatically alters the structure of eukaryotic elongation factor 2 (EF2), affects the actin cytoskeleton [66], and activates nucleases, resulting in apoptosis. Much of the DT that enters the cell is degraded in the lysosomal pathway. In cases where the extracellular fluid is acidic, DT may be directly transported across the plasma membrane to the cytoplasm.

Diphtheria toxin is very potent; once internalized within a cell, a single molecule of DT appears to be sufficient to induce cell death [67]. However, susceptibility to DT varies across species and cell types and depends on expression of the DTR and affinity of the DTR for DT [62]. Cells of humans and mice differ greatly in their vulnerability to DT, which is a consequence of species-specific differences in the amino acid sequence of HB-EGF that endows the human version with a ~1000× higher affinity for DT than mouse version [23]. Richard Palmiter and other genetic engineers took advantage of these features to generate mice that express the full coding region of the human *DTR* gene (*hDTR*) in specific cell types in mice [23, 24]. *hDTR* expression is driven by gene regulatory elements that are specific to a given cell type. Such mice then receive systemic injection(s) of DT. Since DT interacts very weakly

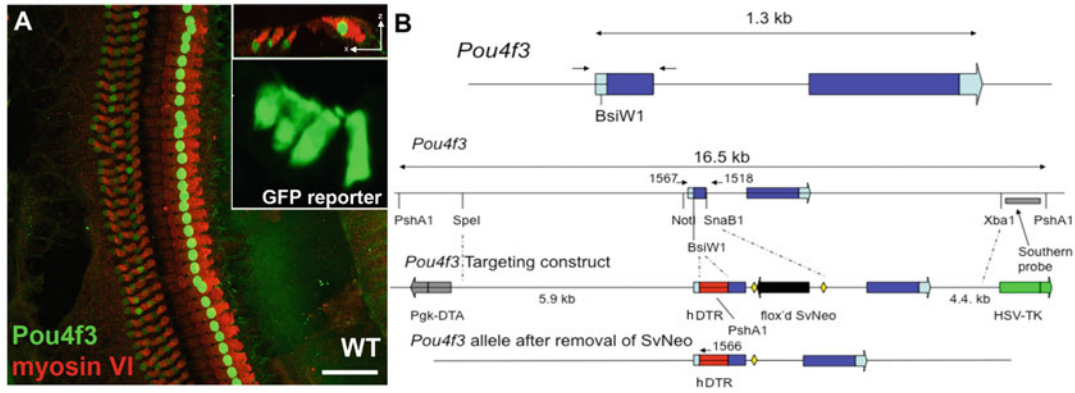

Fig. 1 Strategy for generating *Pou4f3*$^{DTR/+}$ mice. (**a**) Pou4f3 expression is limited to hair cells in the inner ear, as verified two ways: by labeling whole-mount tissue with the Pou4f3 antibody and using sectioned tissue from the *Pou4f3*^{GFP} reporter mouse. Pou4f3 immunolabel (main panel, green) is selectively expressed in the nuclei of hair cells reacted for myosin VI (red) from a mature (P56) WT mouse. An orthogonal view from the same tissue is shown in the upper inset. The *Pou4f3*^{GFP} reporter mouse demonstrates expression in both inner and outer hair cells (lower inset). Scale bar, 50 μm. (**b**) *Pou4f3*$^{DTR/+}$ mice were genetically engineered to contain the human *DTR* downstream of the *Pou4f3* promoter, creating a mouse model in which sensory hair cells in the inner ear can be selectively ablated after a systemic injection of DT. From [58]

with mouse HB-EGF, this treatment has only minor effects on most cells of these mice. However, cells that express *hDTR* quickly undergo cell death.

To target hair cells for DT-induced ablation, *hDTR* was inserted into exon 1 of the *Pou4f3* gene, generating *Pou4f3*^{DTR} mice (Fig. 1b; [58, 59, 60, 68]). Pou4f3 is a transcription factor that, at the protein level, is highly expressed in the nucleus of all inner ear hair cells (Fig. 1a), at early and late stages of development and in maturity but is not expressed in other key cell types in the auditory or vestibular periphery such as supporting cells or primary sensory neurons [69–71]. This *hDTR* insertion inactivates the *Pou4f3* coding region. Germline knockout mice that are homozygous null for *Pou4f3* experience hair cell death in the early postnatal period [69, 71]. By contrast, mice heterozygous for *Pou4f3* exhibit apparently normal development of cochlear hair cells, vestibular hair cells, and hearing [72]. Therefore, *Pou4f3*^{DTR} mice are used as heterozygotes.

A related—but very different—approach to cell killing is to express an inducible form of the gene for the DT-A fragment within a specific cell population (e.g., [73, 74]). This approach has been used to kill supporting cells (e.g., [75]) and hair cells (e.g., [44, 45]) in the mouse inner ear. Another similar approach has been to use mice with Cre-inducible *DTR* expression (e.g., [76]), killing cochlear ganglion neurons.

The first studies to implement the *Pou4f3*^{DTR} mouse line [58, 59, 60, 68] sought to destroy all hair cells in the cochlea and vestibular organs of neonatal and adult mice, in order to assess changes in cellular properties in the cochlear nucleus, hair cell regeneration in the vestibular epithelium, or development of vocalizations in mice.

4 Auditory Hair Cell Ablation Using Pou4f3^{DTR} Mice

Figure 2 shows phenotypic responses of mature *Pou4f3*$^{DTR/+}$ mice given single IM injections at a dosage of 25 ng/g of diphtheria toxin [58]. A single intramuscular (IM) injection of DT (25 ng/g) is sufficient to kill all cochlear hair cells (Fig. 2a–d). Hair cell loss is evident within 3 days of DT injection. At 5 days after DT treatment at this dosage, no normal-appearing hair cells remain. Assessment of cochlear function by auditory brainstem response (ABR) thresholds indicates that these DT-treated mice fail to show any reliable response to clicks or pure tone stimuli up to 90 dB (SPL) by 5 days after the DT treatment (Fig. 2e). None of these pathologies are seen in wild type mice from the same litters given identical injections of DT nor in *Pou4f3*$^{DTR/+}$ mice given saline injection. Of importance, both qualitative and quantitative analyses indicate that the cochlear pathologies resulting from the DT injection at this dosage (or below) appear entirely specific for hair cells. Other cell types of the cochlea, such as epithelial supporting cells, lateral wall fibrocytes, cells of stria vascularis, and spiral ganglion cells appear unaffected [58, 59, 68, 77]. Interestingly, during the period of hair cell loss, there appears to be a small but significant reduction of the endocochlear potential (EP) that subsequently recovers to normal levels. DT dosage, survival period, and age of injection were also varied by Tong et al. and Kaur et al. Complete loss of inner and outer hair cells is also observed in mature *Pou4f3*$^{DTR/+}$ mice given single injection dosages of 15 ng/g and 5 ng/g, but the timing of hair cell loss is delayed by up to 5 days. For the use of *Pou4f3*$^{DTR/+}$ mice to study the influence of synaptic activity on development of brain structure and function, it is critical to quantify neural activity in the neurons under investigation. Studies cited above lead to the assumption that auditory nerve activity will be severely diminished (*see* also ref. 78), and the expected dramatic reduction of spontaneous activity has been confirmed in young animals in studies conducted in the laboratory of Prof. Rudolf Rubsamen in Leipzig (Fig. 2f). At 6 days after DT injection, spontaneous discharge in AVCN neurons was reduced by >99%. More extensive studies on the changes on ongoing ("spontaneous") activity throughout the auditory pathways in neonatal and mature *Pou4f3*$^{DTR/+}$ mice are needed. Recent developments in methods for in vivo Ca^{2+} imaging should facilitate this.

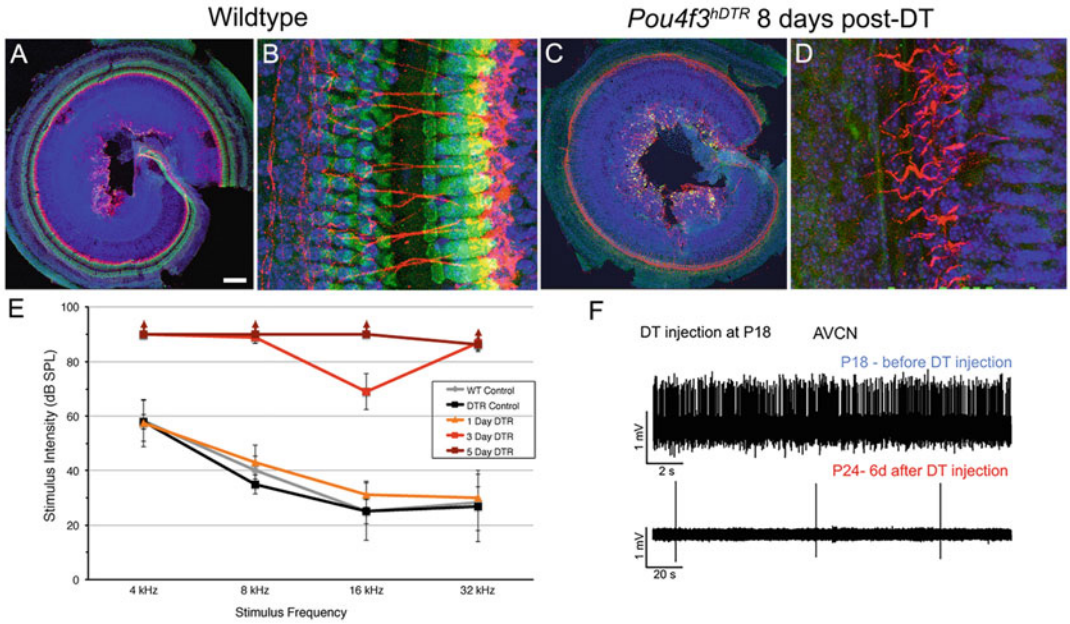

Fig. 2 Summary of auditory phenotype of *Pou4f3*$^{DTR/+}$ mice following DT treatment. (**a**–**d**) Low and high magnification confocal images of mature cochleas from wild type (WT) mice (**a**, **b**) and *Pou4f3*$^{DTR/+}$ mice at 8 days following IM injection of 25 ng/g DT (**c**, **d**). Tissue is reacted for myosin 7a (green), neurofilaments (red), and nuclei (blue). Note complete loss of all hair cells and robust survival of nerve fibers in **c** and **d**. Scale bar in **a** = 100 μm. **b** and **c** from [58]. (**e**) Average (+/− SEM) auditory brainstem response (ABR) thresholds from WT, mice and *Pou4f3*$^{DTR/+}$ mice at 1–5 days after DT treatment. Note total loss of response by 5 days. From [58]. (**f**) Electrophysiological recordings from single neurons in the antero-ventral cochlear nucleus (AVCN) of a young *Pou4f3*$^{DTR/+}$ mouse before and 6 days after 15 ng/g IM injection of DT. Note the difference in time scale on *x*-axis. Action potential frequency is reduced by over 99% by 6 days after DT injection. Data provided courtesy of Prof. Rudolf Rubsamen, Univ. of Leipzig

In summary, the studies cited above indicate that the *Pou4f3*$^{DTR/+}$ mouse appears to be an excellent experimental model for quickly eliminating cochlear hair cells and hearing function, with minimal damage to other cell types. This technique has now been employed in a number of studies, with highly consistent outcomes (e.g., [79, 80]).

As noted above, an important aspect of the *Pou4f3*$^{DTR/+}$ model is the ability to eliminate hair cells at any postnatal age. Several studies have used this property for studies of organ of Corti hair cell regeneration and the effects of hearing loss in neonatal vs mature mice (e.g., [44, 58, 68, 81]). Results of DT injections have been similar to those noted above with a couple of exceptions. First, smaller injections of DT have typically been used (<10 ng/g). Unpublished experience revealed considerable lethality with doses >15 ng/g. With P2–P7 mice, a single dose of 5 mg/g yields complete hair cell loss within 10 days and minimal but detectable rapid changes in organ of Corti supporting cells. On the other

hand, the response of spiral ganglion cells was profoundly different than mature mice. When neonatal *Pou4f3*$^{DTR/+}$ mice were injected with DT, they showed profound loss of SGNs as early as 8 days later. This loss progressed such that only 30% of spiral ganglion cell bodies remained 70 days later. Wild type mice injected with DT as neonates did not have any observable SGN cell body loss at any time point [58]. Finally, the neonatal mouse cochlea can be explanted and maintained in organotypici culture. Hair cells in explanted cochleae from *Pou4f3*$^{DTR/+}$ mice can be selectively lesioned by adding DT to the culture medium. Treating such cultures for 3 days with 25 ng/ml DT results in complete loss of inner hair cells, extensive loss of outer hair cells, and survival of supporting cells [58]. Much more detailed and quantitative work needs to be conducted on the use of this model for in vitro studies.

5 Vestibular Hair Cell Ablation Using Pou4f3^{DTR} Mice

Golub et al. [60] sought to destroy all hair cells in the vestibular organs of adult mice (6–9 weeks of age) and then assess subsequent hair cell regeneration over time. This study employed *Pou4f3*$^{DTR/+}$ mice in order to overcome the limitation of partial hair cell ablation that was observed with all prior methods. For studies of hair cell regeneration, it is advantageous to kill all original (native) hair cells, so that any newly produced hair cells can be definitively identified as "replacement" or "regenerated." In contrast, an incomplete hair cell lesion leaves open the possibility for cellular repair or migration of surviving hair cells into the injured area, both of which would confound data interpretation. Therefore, Golub et al. [60] implemented *Pou4f3*^{DTR} mice, with the goal of killing all vestibular hair cells and preserving other cell types. Hair cells were counted in several control mice to assess the specificity of the method. Analysis of utricular hair cell numbers in *Pou4f3*$^{DTR/+}$ mice that did not receive DT injection revealed that vestibular hair cells develop normally in *Pou4f3* heterozygotes [60]. In addition, injection of DT (two intramuscular injections of 25 ng/g, 2 days apart) to *Pou4f3*^{DTR} wild type mice (*Pou4f3*$^{+/+}$) failed to cause hair cell loss. This finding was expected because the low dose of DT should not induce hair cell loss in mice lacking the *hDTR*. However, in *Pou4f3*$^{DTR/+}$ mice, the same DT regimen caused 50% of vestibular hair cells to die by 7 days post-DT and 94% of hair cells to die by 14 days post-DT. A few papers have demonstrated that DT induces apoptosis-like death of vestibular hair cells (e.g., [82, 83]). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) at 7 days post-DT revealed wide-spread chromatin degradation characteristic of apoptosis in cells throughout the utricle (Fig. 3a, b). Condensed chromatin could be detected using 4′,6-diamidino-2-phenylindole (DAPI) labeling in small numbers of hair cells as

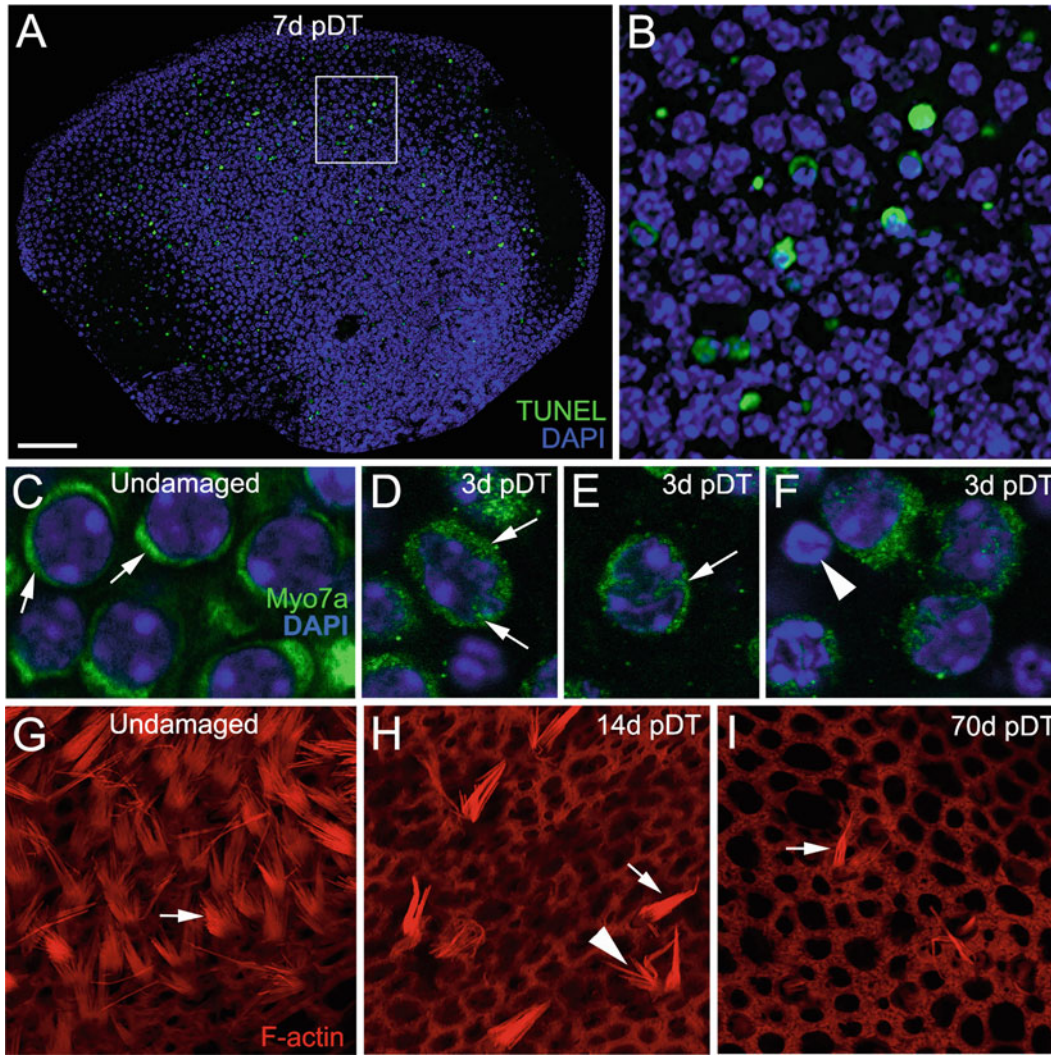

Fig. 3 Hair cell degeneration and death in adult mouse utricles following DT administration. (**a**) TUNEL labeling in a whole-mount utricle from a *Pou4f3*$^{DTR/+}$ mouse at 7 days post-DT. (**b**) Higher magnification of the box shown in **a**. (**c**) Undamaged WT hair cells (arrows) with cytoplasm labeled green (Myosin 7a) and nuclei labeled blue. (**d**)–(**f**) Utricle with similar labeling as **c**, showing hair cells (arrows) from *Pou4f3*$^{DTR/+}$ mice at 3 days post-DT. Arrowhead in F points to nucleus of an apoptotic cell. (**g**) Phalloidin labeling of filamentous (F) actin in stereocilia (arrow) in an undamaged WT utricle. (**h**, **i**) Stereocilia of surviving hair cells (arrows) at 14 (**h**) and 70 (**i**) days post-DT in *Pou4f3*$^{DTR/+}$ mice. Arrowhead in (**h**) shows a bundle of abnormally splayed stereocilia, indicative of injury. The stereocilia of regenerated hair cells are too small and too lightly labeled in panel **i** at 70 days post-DT to be evident. Scale bar: 100 μm in **a**, 12 μm in **b**, 4 μm in **c**–**f**, and 20 μm in **g**–**i**

early as 3 days post-DT (Fig. 3f). Imaging with transmission electron microscopy also provided evidence for pyknotic nuclei at 7 days post-DT (not shown). Additional pieces of evidence of hair cell demise were: (1) deformation of the nucleus; (2) ectopic distribution of myosin 7a (Myo7a) protein, which is normally

cytoplasmic (Fig. 3c) but in some cells appeared to penetrate the nucleus (Fig. 3d–f); and (3) degeneration of stereocilia in the apical hair bundle (Fig. 3g–i).

Amniotes have two types of vestibular hair cell—type I and type II (reviewed in [84]). Mouse utricles contain approximately 3800 hair cells, about half of which are type I [85, 86]. Golub et al. [60] showed that ~200 hair cells of both types remained at 14 days post-DT, but over the next several months, cells resembling type I hair cells decreased further in number. In contrast, hair cells with type II characteristics increased in number to approximately 700 by 60 days post-DT, after which their numbers remained the same. These observations are consistent with the hair cell replacement noted in mice or guinea pigs after hair aminoglycoside treatment, indicating the *Pou4f3*^{DTR} mouse model is a viable tool for regeneration research.

The delay of hair cell destruction, particularly in type I hair cells, indicated that DT uptake, DT trafficking, and/or execution of cell death takes place over several weeks. The design logic for *Pou4f3*$^{DTR/+}$ mice predicted that DT administration should rapidly kill hair cells in adult mice. Further, it was anticipated that all hair cells in adult mice would die rapidly because they continue to express high levels of Pou4f3 (*see* Fig. 1). The finding that 6% of utricular hair cells remained in *Pou4f3*$^{DTR/+}$ mice at 14 days post-DT was surprising, and there are several possible interpretations. First, some of these hair cells may have already been regenerated. This interpretation is supported by the observation that many hair cells at this time lacked a well-formed bundle, which is a sign of immaturity. However, this interpretation cannot account for all remaining hair cells, because many of them were type I and are not naturally regenerated in adult mammals (e.g., [21, 22, 46, 82, 87]). A second possibility is that *Pou4f3* promoter activity may vary amongst hair cells, causing some cells to have insufficient human *DTR* expression, which would result in DT resistance. In other mouse lines employing a similar strategy, complete cell death is obtained in a shorter period. For instance, Wu et al. [88] killed 99% of neurons expressing agouti-related protein within 6 days. Third, DT may be processed differently by similar cell types. For instance, DT enters the cell via endosomes and may remain in that compartment longer in some cells, which would protect them from DT's lethal inhibition of protein translation.

DT kills hair cells in other vestibular organs beside the utricle. Hicks et al. [87] found that, in addition, hair cells were killed, and type II hair cells were regenerated in the anterior and lateral ampullae and in the saccule. It is not clear from studies if there are any spatial gradients in hair cell loss within the vestibular organs.

The degree and nature of hair cell loss in utricles of adult mice can be reduced by administering DT at lower overall doses. A single injection of DT at 25 ng/g induces less extensive hair cell loss in the

utricle [77], seemingly inducing a comparable amount of type II hair cell death and regeneration but sparing approximately half of the type I hair cell population (J. Stone, unpublished data).

Golub et al. [60] also used DT to kill vestibular hair cells in organ cultures. They found that, in whole utricles that were explanted and incubated at 37 °C, overnight incubation with DT (dissolved in culture media at 3–333 ng/ml) destroyed 99% of hair cells in adult utricles within 5 days. These tests were intended as a demonstration of efficacy of the mouse model; systematic dosage and timing studies are needed to make this model more useful for studies in cultured inner ear organs. There are important considerations for studying hair cell damage and regeneration ex vivo. For instance, Lin et al. [89] showed there is substantial spontaneous death of hair cells in cultures even in the absence of a damaging agent presumably due to stress and malnutrition in those conditions, and hair cell regeneration is thwarted in whole cultured utricles, which can only be maintained for 3–4 weeks before tissue undergoes degeneration.

Vestibular hair cells of neonatal *Pou4f3*$^{DTR/+}$ mice can also be lesioned by systemic DT treatment. Much lower dosages are used in neonates. For instance, a single 5 ng/g DT injection kills ~80% of hair cells in the cristae of the semicircular canals (M. Warchol, unpublished data). However, this dose is highly toxic to the sensory epithelium of the neonatal utricle. Treatment of neonates with 5 ng/g DT at P0-1 results in a large epithelial "wound" in the central region of the utricular sensory epithelium, which is caused by the loss of *both* hair cells and supporting cells [90]. Such wounds are apparent at 7 days post-injection, and lead to the mixing of the inner ear fluids (perilymph and endolymph). Such extensive damage also results in the death of the majority of afferent neurons. Interestingly, such wounds begin to close between 7 and 14 days post-DT, probably via the contraction of a "purse-string" actin ring that surrounds the outer border of the wound [90]. These unexpected findings point to an epithelial repair process that is present in the neonatal utricle, similar to that described by Meyers and Corwin [91] after induction of small "punch" wounds in organotypic cultures.

6 Methods

6.1 Mouse Breeding

To drive expression of *hDTR* in mouse hair cells, the gene for human HB-EGF was inserted into exon 1 of *Pou4f3* in the mouse genome (Fig. 1). Mice that are heterozygous for this allele retain one functional copy of *Pou4f3*, and their auditory and vestibular hair cells appear to develop normally [58, 60, 69–71]. However, mice that possess two copies of *hDTR* lack a functional *Pou4f3* gene, and such mice exhibit a profound loss of cochlear and

vestibular hair cells after they are formed during development. For this reason, an optimal breeding strategy for studies targeting hair cell damage in an otherwise physiologically normal animal is to mate *Pou4f3*$^{DTR+/-}$ mice to WT mice, which will yield 50% of offspring that are *Pou4f3*$^{DTR+/-}$ ("experimental" mice) and 50% that are WT and can be used as controls.

For genotyping, DNA is extracted from mouse tissue (typically via tail-clip), polymerase chain reaction amplifies a portion of the *Pou4f3* gene that is either WT or contains the *hDTR* allele, and gel electrophoresis is used to distinguish between these two gene segments, which differ in size. Detailed methods for genotyping these mice can be found in Tong et al. [58] and Kaur et al. [77].

Genetic background is important to consider when breeding. Most published studies have utilized C57Bl6/J mice, in which consistent lesions have been achieved. However, González-Garrido et al. [83] noted that full hair cell lesions were not reliably attained with the standard DT dose (25 ng/g) in adult *Pou4f3*$^{DTR+/-}$ mice on a CBA/CaJ background. This same result was experienced by the University of Washington team, and a single cross of congenic CBA-CaJ mice to C57Bl6/J mice restored the sensitivity of the mice to DT (unpublished observations).

6.2 DT Administration

Diphtheria toxin in unnicked form is injected either intramuscularly (IM) to juvenile and adult mice or intraperitoneally (IP) to neonatal mice. While some investigators report intraperitoneal (IP) injections in neonatal mice, in our hands, both IM and subcutaneous injections in neonates can lead to unreliable results likely due to leakage of the solution from the injection site. With this approach, we have attained nearly symmetric hair cell lesions in organs from the left and right sides of the mouse, and we have achieved similar lesions in all mice from a given cohort.

Monaural DT treatment, causing unilateral hair cell destruction, would be useful for both inner ear and CNS studies. Pilot studies were undertaken in the Rubel lab to determine if local injections of DT into the middle ear of mature mice will produce single-sided hair cell loss and deafness. The results were promising but optimal formulations, dosages, and timing need to be resolved.

DT powder is purchased from Sigma-Aldrich #D0564 or List Biological Labs #150 and can be stored at 2–8 °C. DT powder is readily dissolved in water. We make 1 μg/100 stock solution dissolved in saline (0.9% NaCl sterile) solution and store it in a non-defrosting (−20 °C) freezer. The drug is most dangerous in powder form or at high concentrations in solution. People using the drug should be vaccinated and should consult Material Safety Datasheets, standard operating procedures, and university resources (e.g., Occupational Nurse, Environmental Health and Safety) for instructions and assistance in handling.

In the first 3–7 days after injection, mice can react to DT by grouping together and consuming less water and food, but they usually improve by 1 week. Nutritional supplements such as the high-calorie gel Nutri-Cal (Tomlyn/Vétoquinol USA) have been added to cages to keep juvenile or adult mice healthier during the first week post-DT. If they are sick, we administer subcutaneous lactated Ringers solution. Supplements are more likely required when adult mice receive two doses of DT at 25 ng/g or higher.

A higher DT dose is required to achieve full destruction of vestibular hair cells than to induce complete loss of cochlear hair cells in adult mice [58, 60]. To reliably kill all vestibular hair cells, we inject either 25 or 50 ng/g DT (IM), once a day, for 2 days, skipping 1 day between injections. A single injection at either dose typically results in loss of most type II hair cells but only half of the type I hair cell population (J. Stone, unpublished data).

We found that DT's efficacy in hair cell killing can vary lot-by-lot, and DT solution loses its efficacy over months when stored in the freezer. Therefore, we run a dose-response test for each new lot of DT, measuring hair cell loss at 14 days post-DT, and we test the DT solution every 4–6 months, discarding it when it no longer induces a lesion at doses equal or less than 50 ng/g.

Finally, we have observed a small degree of hair cell loss in the cochleae of WT mice after treatment with high doses of DT (25–50 ng/g). This loss is usually confined to IHCs and is very minor when compared to the hair cell death that occurs in *Pou4f3*$^{DTR+/-}$ mice after the same DT treatment. Also, this hair cell loss is not extensive enough to cause elevated ABR thresholds in DT-injected "control" mice (e.g., *see* ref. 58). Still, this effect has the potential to influence the outcomes of certain types of studies and should be carefully monitored. The reason for this small degree of DT-induced cell death in WT mice (which do not possess *hDTR*) is not clear. However, genomic studies indicate that some mouse hair cells express HB-EGF (data publicly available at umgear.org). Furthermore, even though the affinity of DT for human HB-EGF is significantly greater than its affinity for the mouse form of this protein, it is still possible that a small number of DT molecules are transported into hair cells of WT mice. A complete explanation of this phenomena will require further investigation.

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