Morphological and functional analyses of vestibular hair cell loss and replacement in adult Pou4f3^{DTR} mice after treatment with two different doses of diphtheria toxin

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Background and Objectives

In a prior study, we examined vestibular hair cell ablation and regeneration using Pou4f3^{DTR} mice, in which heparin-binding epidermal growth factor (the diphtheria toxin receptor, or DTR) is expressed under control of the *Pou4f3* promoter (Golub et al., 2012). In Pou4f3^{DTR} mice, hair cells are specifically susceptible to diphtheria toxin (DT). In this prior study, we examined changes in hair cell numbers in utricles of adult mice on a C57Bl/6J background following intramuscular injection of 50 ng/g.

Here, our objectives were to examine vestibular hair cell ablation and regeneration in Pou4f3^{DTR} mice on a different background (CBA/CaJ), to include analysis of the lateral ampulla and the vestibular nerve, and to test vestibular function, after administration of two doses of DT - 25 ng/g and 50 ng/g.

Methods

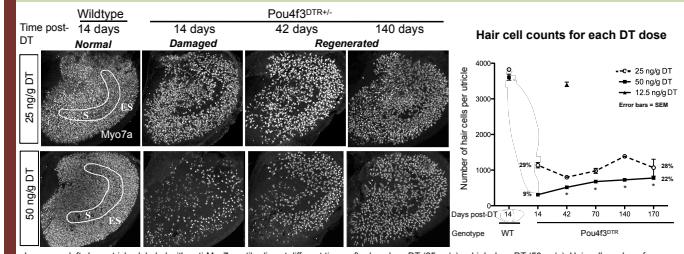
Animals and DT administration: 6-9 week-old Pou4f3$^{DTR+/-}$ mice on a CBA/CaJ background received one of the following: a single IM injection of DT at 12.5 ng/g, a single IM injection of DT at 25 ng/g, or 2 IM injections of DT at 25 ng/g (spaced 2 days apart; a total dose of 50 ng/g). Controls consisted of wildtype mice that received similar injections, or Pou4f3^{DTR} mice that received no DT. Mice were euthanized at 14, 42, 70, 140, or 170 days post-DT, or equivalent (for uninjected mice).

Histological analysis: In utricle whole-mounts, we examined: hair cell and supporting cell counts, neurofilament labeling of fibers in the macula, Ctbp2 and GluR2 labeling of ribbon synapses, and transmission electron micrographs (TEM) of synapses. In lateral ampulla whole-mounts, we examined hair cell counts; analysis of nerves and ribbon synapses is underway. Organs were imaged using confocal microscopy. We assessed vestibular ganglion neurons in sections of plastic-embedded temporal bones.

Vestibulo-ocular reflex (VOR) testing to assess function of lateral ampullae. At different times post-DT, we recorded eye movements in the dark while mice were rotated *en bloc* about a vertical axis at a range of frequencies (0.3, 0.5, 0.7, 1.0 Hz) at a fixed amplitude of 20°. VOR gains (eye velocity/head velocity) were computed for each frequency.

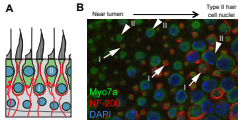
Results

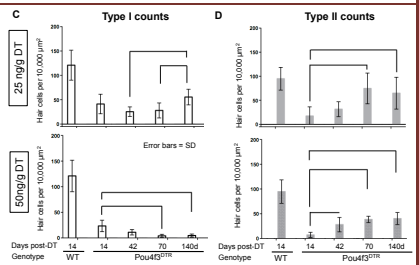
Hair cell loss and regeneration in utricles: DT dose effect



Images on left show utricles labeled with anti-Myo7a antibodies at different times after low-dose DT (25 ng/g) or high-dose DT (50 ng/g). Hair cell numbers for each time and dose are graphed on the right. **% of control** is indicated for some data points. **Initial hair cell loss was greater and faster with 50 ng/g DT than 25 ng/g, but late hair cell counts were similar.** 12 mg/g DT causes minimal hair cell loss (triangle at 42d). *Sig'ly diff from Pou4f3^{DTR} at 14d post-DT.

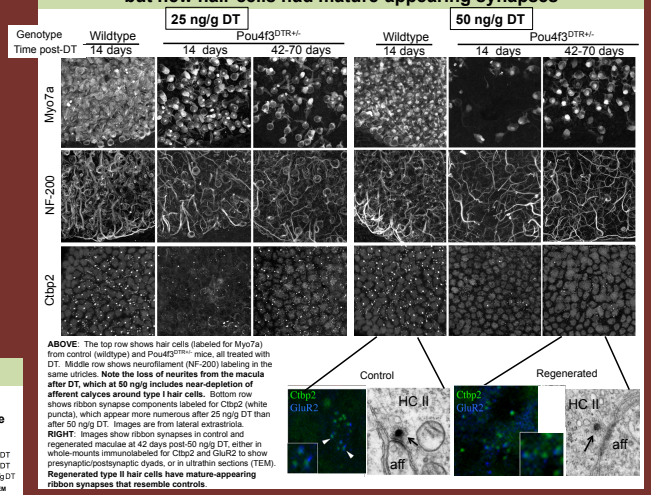
Effects of DT dose on numbers of type I and II hair cells. We counted type I and II hair cells in whole-mounted utricles at different times post-DT in wildtype (WT) and Pou4f3$^{DTR+/-}$ mice. Utricles were labeled with antibodies to Myo7a to detect all hair cells and 200 kD neurofilament (NF-200) to detect calyceal afferent nerves surrounding type I hair cells. **A,B** define the criteria we used to distinguish type I from type II hair cells. **B** is a confocal slice, nearly parallel to the lumen, showing progressively deeper regions of the macula, from left to right. Type I hair cells (arrows in B) have a skinny neck and a neural calyx (red) around the cell body; type II hair cells (arrowheads in B) have a thick neck and no calyx. Counts of each cell type are shown in **C and D** (right). Brackets show groups with p<0.05, ANOVA. All groups were different form controls (WT at 14d post-DT).





Type I hair cells were depleted from utricles after 50 ng/g DT, but persisted and/or were regenerated after 25 ng/g DT. Thus, after 50ng/g DT, type II hair cells were the predominant hair cell type replaced in the utricle.

Neurites and synapses were reduced in utricle after DT treatment, but new hair cells had mature-appearing synapses

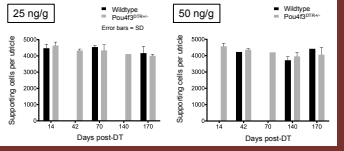


ABOVE: The top row shows hair cells (labeled for Myo7a) from control (wildtype) and Pou4f3$^{DTR+/-}$ mice, all treated with DT. Middle row shows neurofilament (NF-200) labeling in the same utricles. **Note the loss of neurites from the macula after DT, which at 50 ng/g includes near-depletion of afferent calyces around type I hair cells.** Bottom row shows ribbon synapse components labeled for Ctbp2 (white puncta), which appear more numerous after 25 ng/g DT than after 50 ng/g DT. Images are from lateral extrastriola.
RIGHT: Images show ribbon synapses in control and regenerated maculae at 42 days post-50 ng/g DT, either in whole-mounts immunolabeled for Ctbp2 and GluR2 to show presynaptic/postsynaptic dyads, or in ultrathin sections (TEM). **Regenerated type II hair cells have mature-appearing ribbon synapses that resemble controls.**

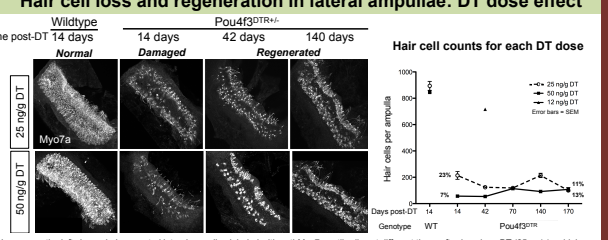
Supporting cell numbers did not change after DT at either dose

We counted supporting cell nuclei in whole-mounted utricles from wildtype and Pou4f3$^{DTR+/-}$ mice treated with 25 or 50 ng/g DT. Utricles were labeled with antibodies to Myo7a to detect all hair cells and DAPI to detect all cell nuclei. Supporting cell nuclei were identified based on their location (near the basal lamina), their relatively small size, and their exclusion of Myo7a (hair cell-specific) labeling.

We found no statistically significant differences in supporting cell numbers between treated Pou4f3$^{DTR+/-}$ mice and wildtype mice over time after either DT dose. Further, there was no change in supporting cell numbers over time after DT in Pou4f3$^{DTR+/-}$ mice. (Age-matched controls - black bars - were analyzed for only some time-points.)

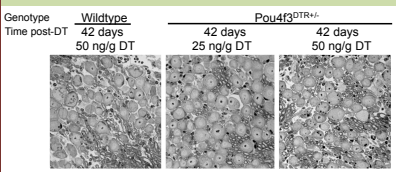


Hair cell loss and regeneration in lateral ampullae: DT dose effect



Images on the left show whole-mounted lateral ampullae labeled with anti-Myo7a antibodies at different times after low-dose DT (25 ng/g) or high-dose DT (50 ng/g). Hair cell numbers for each time and dose are graphed on the right. **Initial hair cell loss is greater and more rapid with 50 ng/g DT than 25 ng/g, but late hair cell counts are similar.** 12 mg/g DT causes minimal hair cell loss (triangle at 42d). **The extent of hair cell replacement is significantly lower in the lateral ampulla than the utricle** (compare with first figure in this poster). Analysis of ampullae with confocal microscopy was technically challenging, so we are currently examining hair cell subtypes, neurites, and synapses in plastic sections.

Vestibular ganglion cells did not appear to change in response to either DT dose within 42 days



We are examining if vestibular ganglion neurons (VGNs) undergo morphological changes and/or die once hair cell death has been induced by DT. These images of VGNs were derived from 2 µm horizontal plastic sections of temporal bones from wildtype and Pou4f3$^{DTR+/-}$ mice at 42 days post-DT, when extensive hair cell loss and some regeneration had occurred. Changes in response to both low- and high-dose DT were examined. **At this survival time, we detected no clear qualitative or quantitative changes in VGNs following DT treatment at either dose relative to controls.** Analysis of later times-points post-DT is in progress.

Functional recovery during regeneration: DT dose effect

Mice have **overt symptoms** following DT treatment that might be influenced by vestibular hair cell loss. These include head-bobbing, hyperactivity, spinning when elevated by tail (by 7 days post-DT), and circling (70-170d post-DT). Qualitatively, these symptoms seem less severe in mice that receive 25 ng/g DT than those that receive 50 ng/g DT.

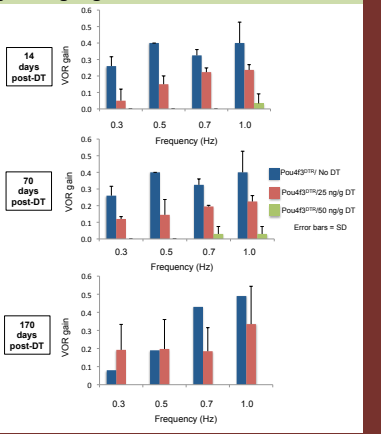
To better assess how hair cell loss and regeneration impact vestibular function, we measured **the gain (eye velocity/head velocity) of the horizontal VOR (vestibulo-ocular reflex) at 4 frequencies for the following sets of Pou4f3$^{DTR+/-}$ mice:**

- **No DT** (age-matched negative controls, with normal hair cell numbers)
- **25 ng/g DT** (moderate hair cell loss, type I and II hair cells present at late stages)
- **50 ng/g DT** (near-complete hair cell loss, only type II hair cells present at late stages)

Mean VOR gains for control mice for each group are represented by **blue bars**.

In mice that received **25 ng/g DT (red bars)**, VOR gains were ~ half of controls at 14, 70, and 170 days post-DT.

In mice that received **50 ng/g DT (green bars)**, there were essentially no rotation-evoked eye movements (gain = 0) immediately after damage (14 days post-DT) or after hair cell regeneration (70 and 170 days post-DT).



Conclusions

This project extended our studies of vestibular hair cell loss and regeneration in Pou4f3$^{DTR+/-}$ mice to examine the lateral ampullla and vestibular afferents in a new mouse background, CBA/CaJ, and to examine two different doses of the damaging agent, diphtheria toxin (DT). We found:

- In utricles and lateral ampullae, hair cell loss was more complete and rapid after 50 ng/g DT than 25 ng/g DT. 12.5 ng/g DT caused little loss of hair cells.
- After treatment with 25 or 50 ng/g DT, we found evidence for vestibular hair cell regeneration. After **50 ng/g DT**, only type II hair cells were regenerated (~20% in utricle, 13% in lateral ampullla). Type I hair cells were depleted from the epithelium by 70 days post-DT. After **25 ng/g DT,** we found evidence for regeneration of both type I and II hair cells. Total hair cell numbers at late survival times were higher after low-dose DT.
- We detected loss of neurites, afferent calyces, and ribbon synapse components from the utricular macula after DT treatment. The degree of loss seemed proportionate to DT dose and hair cell loss, but this remains to be determined. Type II hair cells that were regenerated after 50 ng/g DT had mature-appearing ribbon synapses upon vestibular afferents.
- VOR testing showed that eye movements could not be evoked by head rotation in mice treated with 50 ng/g DT. By contrast, eye movements were elicited by head movements, with modest gains, in mice treated with 25 ng/g DT. If the lateral ampullla is indeed similar to the utricle re. patterns of loss and regeneration of type I and II hair cells, our findings would mean that the VOR is absent when only type II hair cells are present but is present when substantial numbers of type I and type II hair cells are present. Ongoing studies of type I and II hair cells in regenerated lateral cristae will help verify this interpretation.

Acknowledgements and References

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Golub, JS, Tong, L, Nguyen, T, Hume, C, Palmiter, RD, Rubel, EW, Stone, JS (2012). Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. Journal of Neuroscience 32(43):15093-105.