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Figures and figure supplements

Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma

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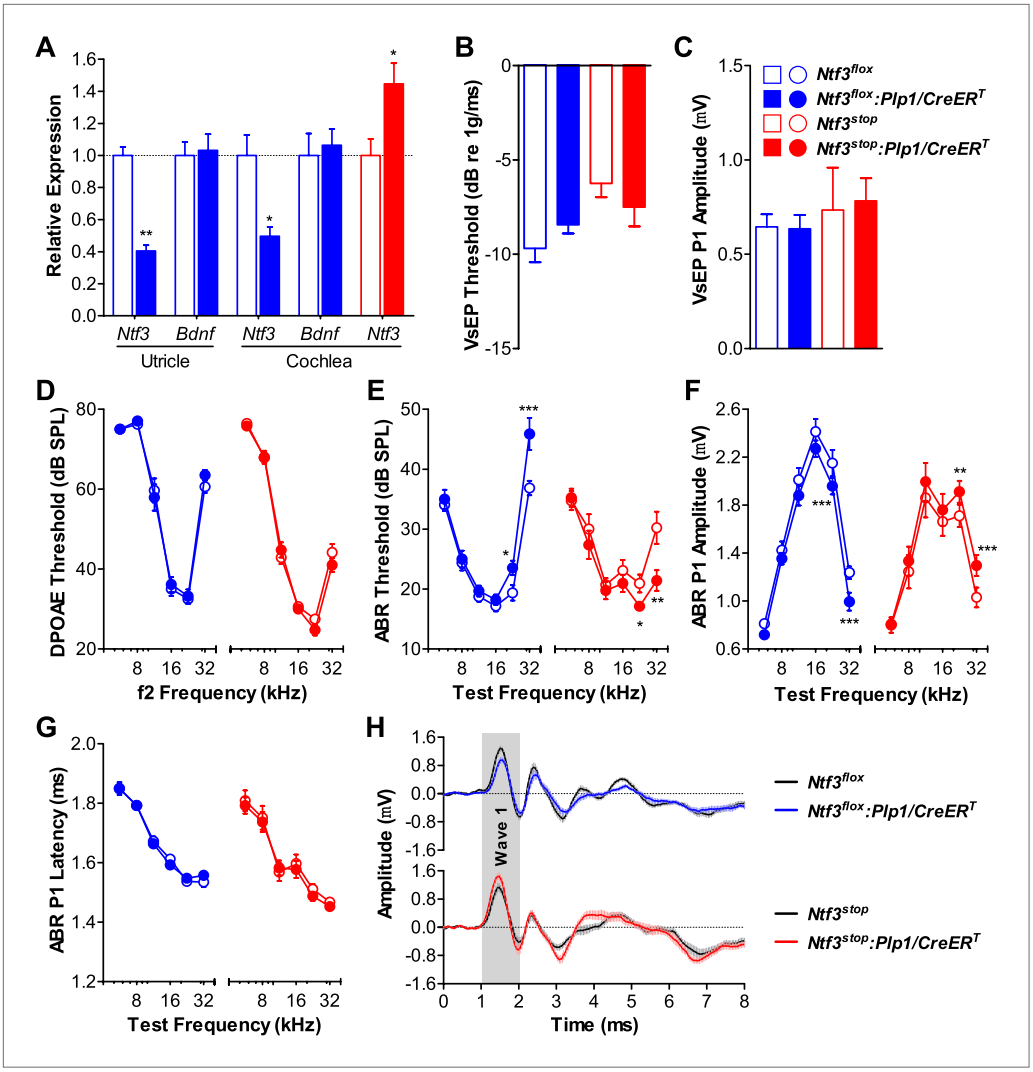

Figure 1. Ntf3 expression by postnatal supporting cells is required for cochlear, but not vestibular function. (**A**) RT-qPCR shows that postnatal tamoxifen injection reduces or increases Ntf3 mRNA levels in adult *Ntf3^{flox}:Plp1/CreER^{T}* or *Ntf3^{stop}:Plp1/CreER^{T}* inner ears, respectively; *n* = 5–6. *p < 0.05, **p < 0.01 by two-tailed unpaired t test. (**B** and **C**) Postnatal *Ntf3* knockout (blue) or overexpression (red) from supporting cells does not alter VsEP thresholds (**B**) or their peak 1 (P1) amplitudes at 0 dB (**C**); *n* = 4–8. (**D**–**F**) Postnatal knockout or overexpression of *Ntf3* from supporting cells reduces or enhances cochlear function, respectively. *Ntf3* knockout (blue) elevates ABR thresholds (**E**) and decreases ABR P1 amplitudes (**F**), without changing DPOAE thresholds (**D**); *n* = 16–17. *Ntf3* overexpression (red) reduces ABR thresholds (**E**) and increases ABR P1 amplitudes (**F**), without changing DPOAE thresholds (**D**); *n* = 21. ABR P1 amplitudes were assessed at 70 dB SPL. *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA. (**G**) ABR P1 latencies are not affected by either *Ntf3* knockout (blue) or *Ntf3* overexpression (red) at all frequencies examined. Key in **C** applies to **A**–**G**. (**H**) Mean ABR waveforms from responses to 32 kHz tone pips from *Ntf3* knockouts and their controls (upper) and *Ntf3* overexpressors and their controls (lower). Gray shading indicates ABR wave 1. Both ABR P1 latencies (**G**) and waveforms (**H**) results were assessed at 70 dB SPL; *n* = 13–17.

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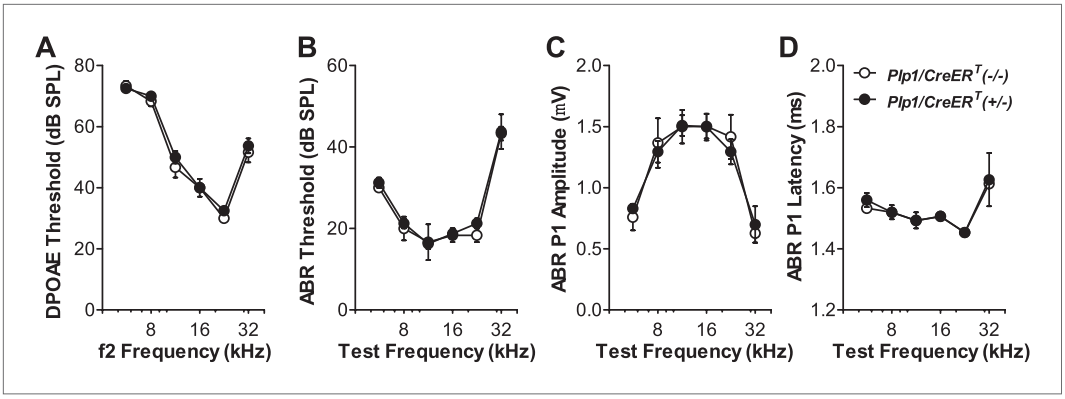

Figure 1—figure supplement 1. Expression of the *Plp1/CreER*^{T} allele does not affect the cochlear function. *Plp1/CreER*^{T}*(+/–)* and *Plp1/CreER*^{T}*(–/–)* littermates exhibit similar DPOAE thresholds (**A**), ABR thresholds (**B**), ABR P1 amplitudes (**C**), and ABR P1 latencies (**D**) at all frequencies tested. ABR P1 amplitudes and latencies were assessed at 70 dB SPL. Tamoxifen was injected at P0-1 and physiological tests were performed at P42; *n* = 4. Key in **D** applies to all panels.

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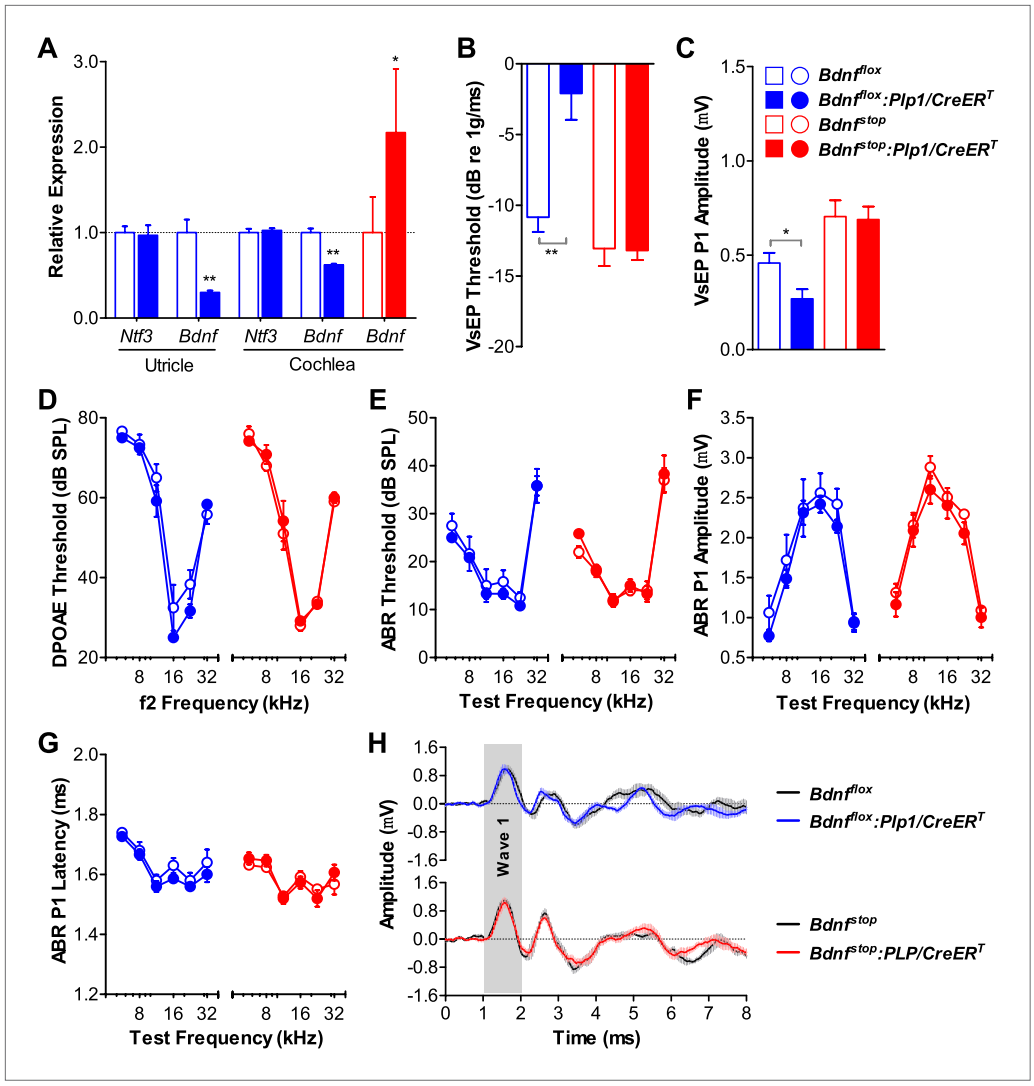

Figure 2. Bdnf expression by postnatal supporting cells is required for vestibular, but not cochlear function. (**A**) RT-qPCR shows that postnatal tamoxifen injection results is reduced or increased Bdnf mRNA levels in adult *Bdnf*^{flox}:*Plp1/CreER*^{T} or *Bdnf*^{stop}:*Plp1/CreER*^{T} inner ears, respectively; *n* = 6. (**B** and **C**) Postnatal *Bdnf* knockout (blue) from supporting cells leads to higher VsEP thresholds (**B**) and lower VsEP P1 amplitudes (**C**) at the high stimulus level (0 dB *re* 1 g/ms). Postnatal *Bdnf* overexpression (red) does not affect the vestibular function (**B** and **C**); *n* = 6–11. *p < 0.05; **p < 0.01 by two-tailed unpaired *t* tests. (**D**–**H**) Neither *Bdnf* knockout (blue) nor overexpression (red) from postnatal supporting cells alters cochlear function, as shown by normal DPOAE thresholds (**D**), ABR thresholds (**E**), ABR P1 amplitudes (**F**), ABR P1 latencies (**G**), and ABR P1 waveforms (**H**) at 70 dB SPL compared to control littermates; *n* = 6. Key in **C** applies to **A**–**G**. Gray shading (**H**) indicates ABR wave 1.

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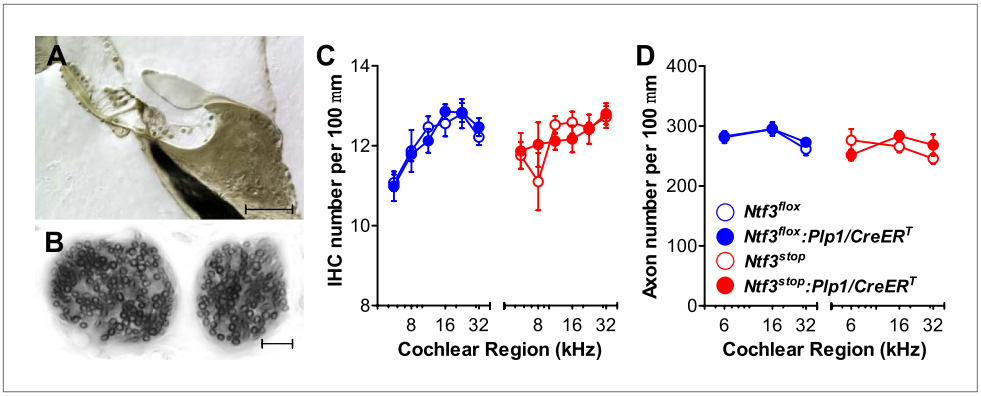

Figure 3. Ntf3 expression by postnatal supporting cells does not affect the organ of Corti morphology, hair cell or axon numbers. (**A** and **B**) Photomicrographs of the basal turn of an *Ntf3*^{flox}*:Plp1/CreER*^{T} cochlea (~32 kHz region) showing a cross-section of the organ of Corti (**A**; scale bar = 100 μm) and a tangential section through the osseous spiral lamina showing peripheral axons of cochlear nerve fibers (**B**; scale bar = 10 μm). (**C**) The number of IHCs per 100 μm of organ of Corti is not altered by either *Ntf3* knockout (blue) or overexpression (red) at any cochlear regions; *n* = 5–6. (**D**) The number of cochlear-nerve peripheral axons per 100 μm of osseous spiral lamina (near the habenula perforata) is not affected by either *Ntf3* knockout (blue) or overexpression (red); *n* = 6. Key in **D** applies to **C** and **D**.

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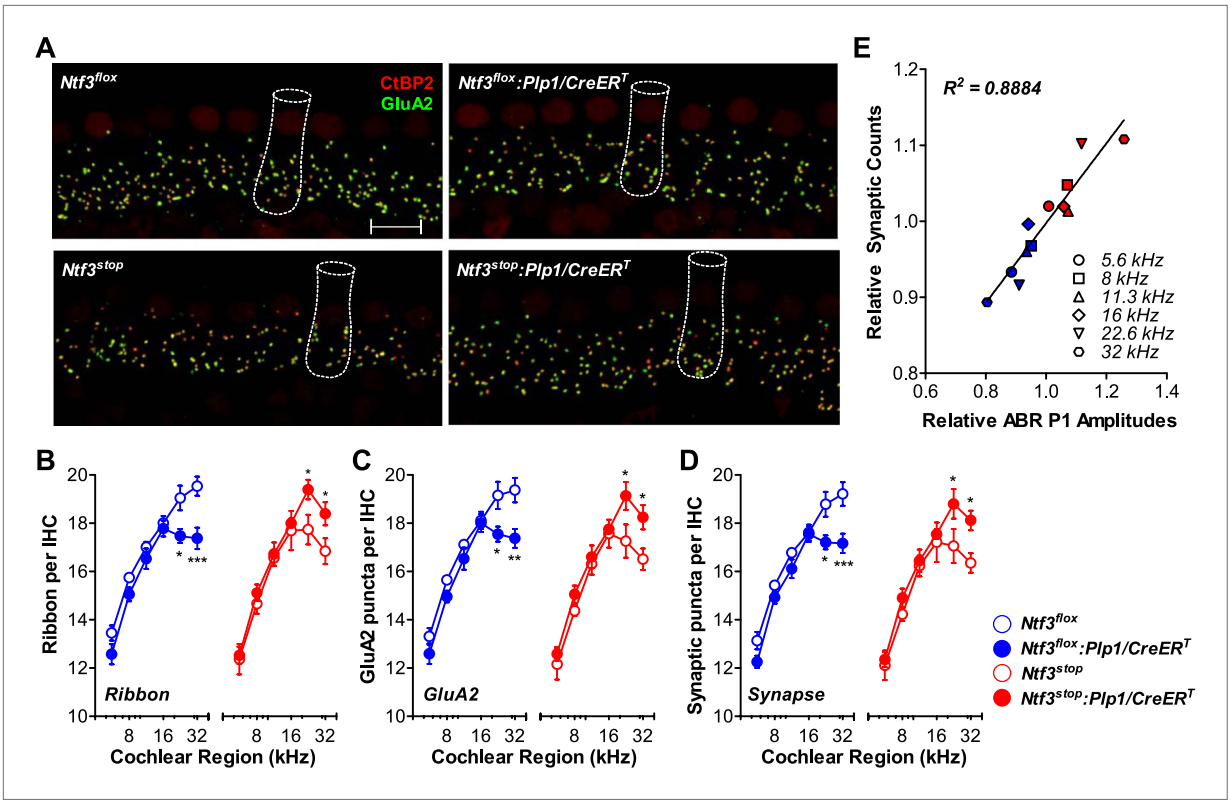

Figure 4. Ntf3 expression by postnatal supporting cells regulates hair cell ribbon synapse density at high frequencies. (**A**) Representative confocal images (maximal projection from a focal series) of IHC synapses from 32 kHz region of *Ntf3*^{flox}, *Ntf3*^{flox}*:Plp1/CreER*^{T}, *Ntf3*^{stop}, and *Ntf3*^{stop}*:Plp1/CreER*^{T} cochleae immuno-labeled for pre-synaptic ribbons (CtBP2-red) and post-synaptic receptor patches (GluA2-green) (scale bar = 10 μm). The dashed lines show the approximate outline of one IHC. CtBP2 antibody also weakly stains IHC nuclei. (**B**–**D**) Quantitative data shows that *Ntf3* knockout reduces, and overexpression increases, the number of pre-synaptic ribbons (**B**), post-synaptic GluA2 receptor patches (**C**), and putative ribbon synapses, defined as juxtaposed CtBP2- and GluA2-positive puncta (**D**) at high frequency cochlear regions; $n = 5–6$. *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA. (**E**) Relative synaptic counts vs relative ABR P1 amplitudes of *Ntf3* knockouts (blue) or overexpressors (red) shows a linear correlation. Data points were obtained by normalizing synaptic counts and ABR P1 amplitudes of *Ntf3* mutants to the values of their respective controls at each of the frequency regions analyzed. Key in **D** applies **B**–**E**.

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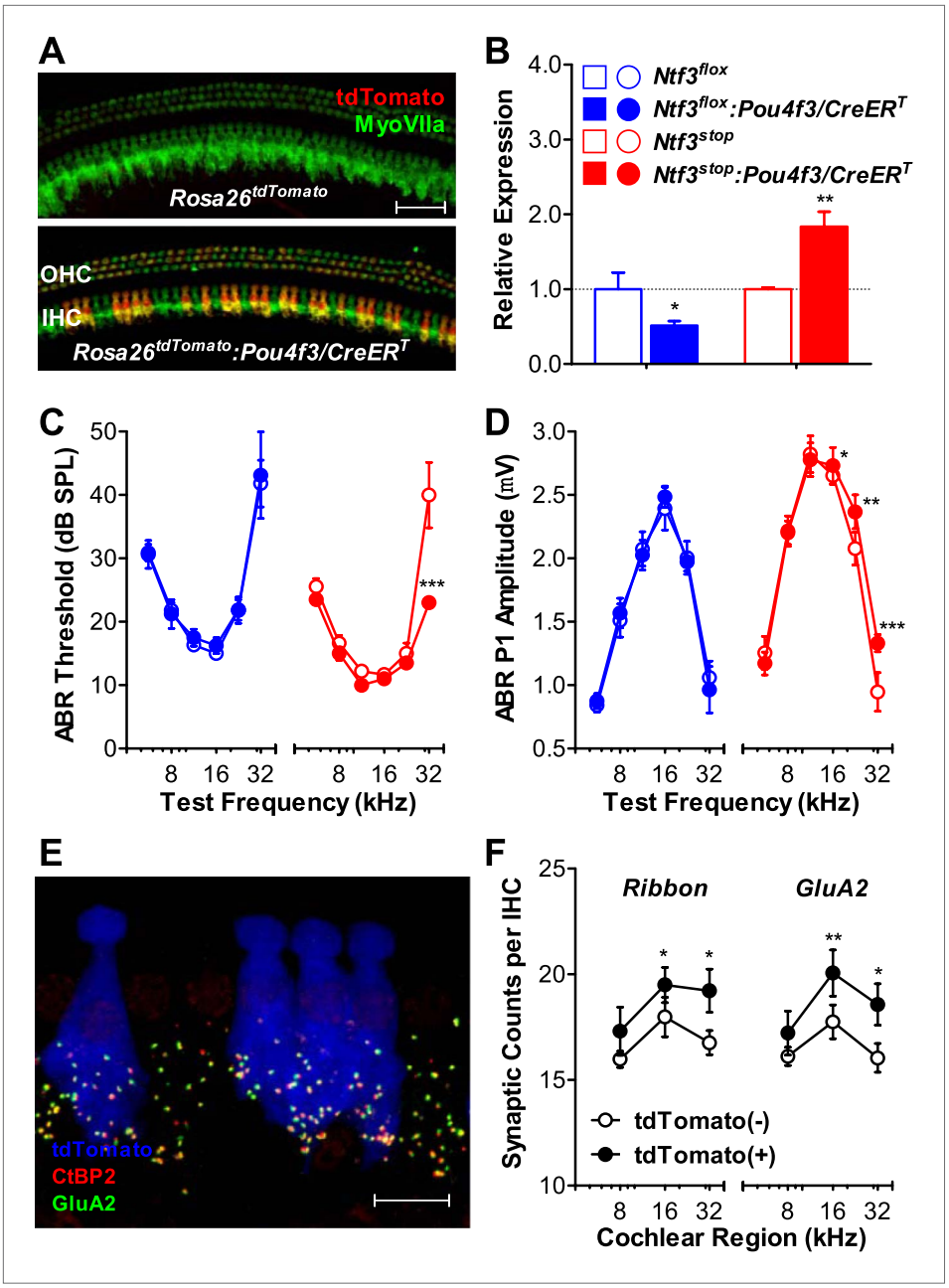

Figure 5. Ntf3 overexpression by postnatal hair cells increases cochlear sensitivity and synaptic densities at high frequencies. (**A**) *Pou4f3/CreER*^{T} allows for hair cells specific inducible gene recombination. *Rosa26*^{tdTomato}*:Pou4f3/CreER*^{T} mice and their littermate *Rosa26*^{tdTomato} controls were injected with tamoxifen at P1-P3, and the cochleas were collected at P60. Inducible recombination is seen as tdTomato fluorescence co-localized with MyoVIIa immunostaining. Scale bar = 50 μm. (**B**) RT-qPCR shows that postnatal tamoxifen injection reduced Ntf3 mRNA in *Ntf3*^{flox}*:Pou4f3/CreER*^{T} cochlea and increased Ntf3 expression in *Ntf3*^{stop}*:Pou4f3/CreER*^{T} cochlea, compared to their respective controls; *n* = 4–5. *p < 0.05, **p < 0.01 by two-tailed unpaired *t* tests. (**C** and **D**) Postnatal overexpression of *Ntf3* in hair cells (red) reduced ABR thresholds (**C**) and increased ABR P1 amplitudes (**D**) at high frequencies; *n* = 9–10. Postnatal knockout of *Ntf3* from hair cells (blue) had no effect on these measures; *n* = 8–11. ABR P1 amplitudes were assessed at 70 dB SPL. *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA. (**E**) Confocal maximal projection of 7 adjacent IHCs from the 32 kHz region of a hair cell-specific *Ntf3* overexpressor, after immunostaining for synapses as in ***Figure 4A***; tdTomato indicates recombined hair cells. Scale bar = 10 μm. (**F**) Synaptic counts are increased in recombined (tdTomato+, *Ntf3* overexpressing) IHCs compared to neighboring unrecombined cells; *n* = 6 cochleae, with at least 100 hair cells in each group. *p < 0.05, **p < 0.01 by two-tailed paired *t* tests. Key in **B** applies to **B**–**D**.

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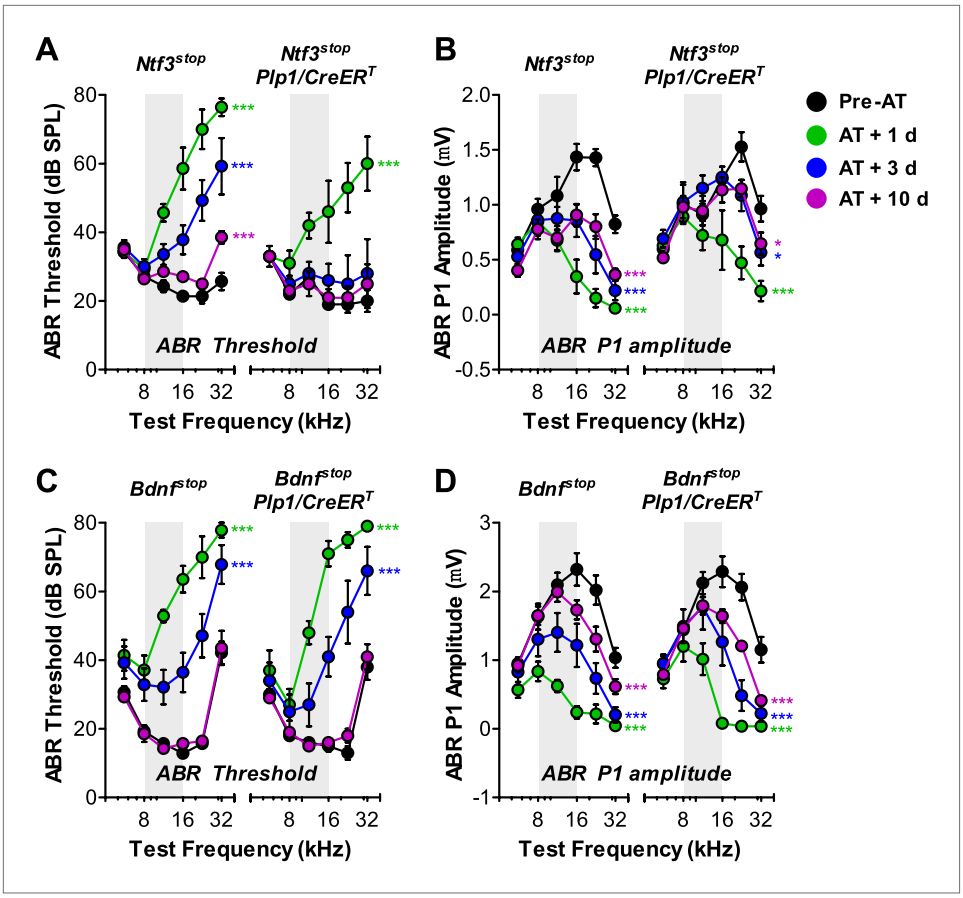

Figure 6. Overexpression of Ntf3, but not Bdnf, promotes recovery from noise-induced attenuation of cochlear responses. (**A** and **B**) *Ntf3* overexpression accelerates the recovery of ABR thresholds (**A**) and promotes recovery of ABR P1 amplitudes (**B**) after acoustic trauma (AT); *n* = 5–7. (**C** and **D**) *Bdnf* overexpression does not affect the recovery of ABR thresholds (**A**) and ABR P1 amplitudes (**B**) after acoustic trauma; *n* = 5–7. *p < 0.05, ***p < 0.001 by two-way ANOVA. Gray shading indicates the noise exposure spectrum. Key in **B** applies to all panels.

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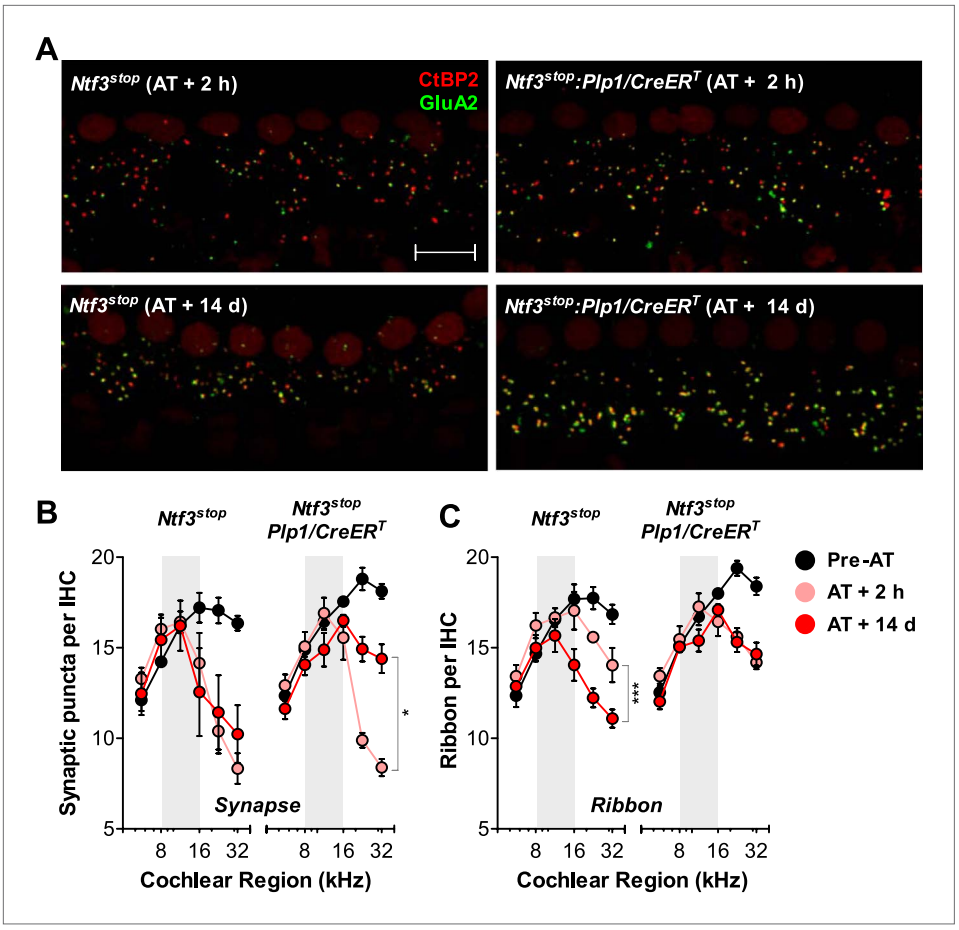

Figure 7. Ntf3 overexpression from postnatal supporting cells promotes recovery from noise-induced synaptic degeneration. (**A**) Representative confocal images of IHC synapses from 32 kHz region of *Ntf3*^{stop} and *Ntf3*^{stop}*:Plp1/CreER*^{T} cochleae immunolabeled for CtBP2 and GluA2 at 2 hr (upper panels) or 14 days after acoustic trauma (AT); scale bar = 10 μm. (**B** and **C**) *Ntf3* overexpression promotes regeneration of IHC synapses (**B**) and prevents the progressive loss of IHC ribbons (**C**) between 2 hr (AT + 2 hr) and 14 days (AT + 14 days) after acoustic trauma. *n* = 3–8. *p < 0.05, ***p < 0.001 by two-way ANOVA. Gray shading indicates the noise exposure spectrum. Key in **C** applies to **B**–**C**.

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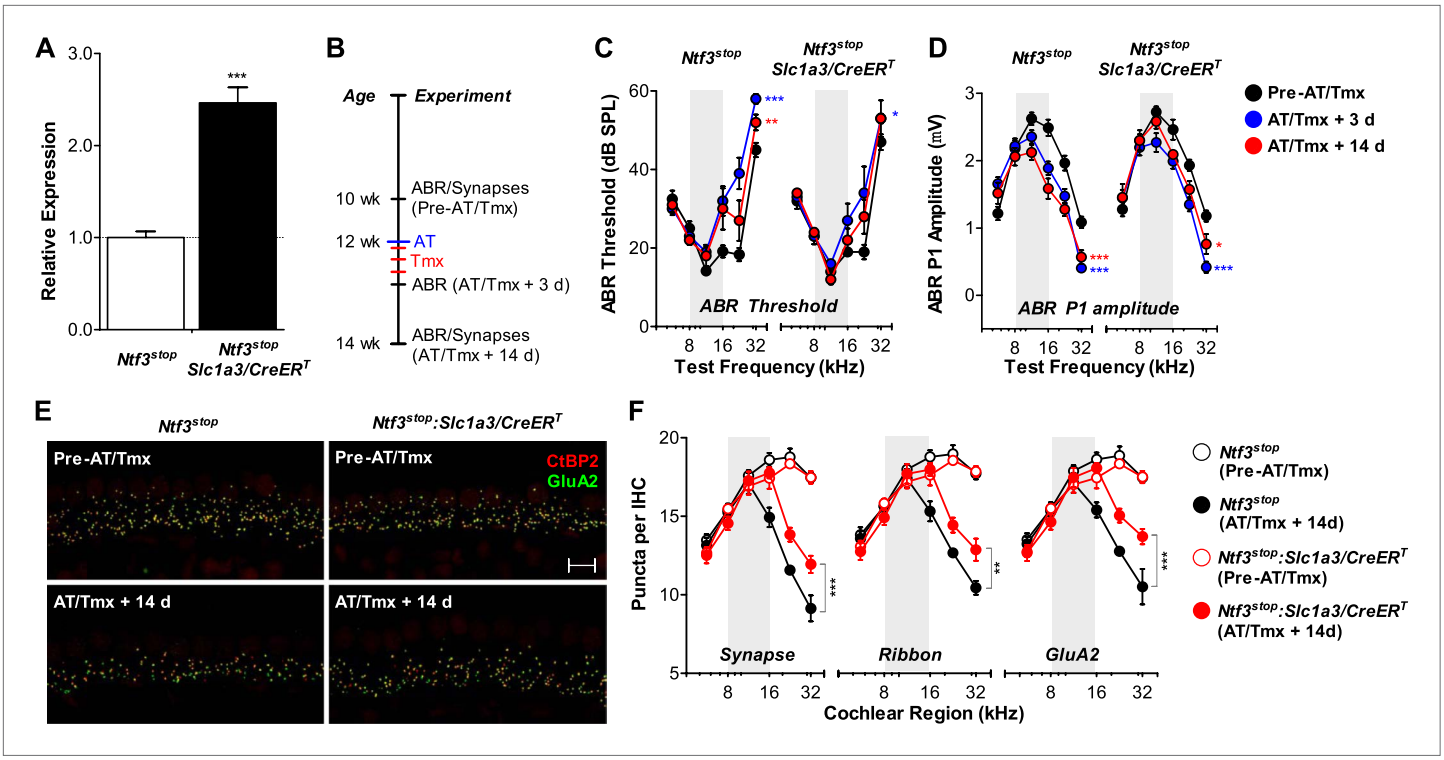

Figure 8. Ntf3 overexpression from adult supporting cells after acoustic trauma promotes auditory function recovery and synaptic regeneration. (**A**) RT-qPCR shows that tamoxifen treatments of adult mice increased Ntf3 expression in *Ntf3^{stop}:Slc1a3/CreER^{T}* cochlea; *n* = 6. ***p < 0.001 by two-tailed unpaired *t* tests. (**B**) Time line of the experiment showing the ages of mice for ABR measurements, acoustic trauma (AT), tamoxifen inductions (Tmx), and sample collections for synaptic counts. (**C**–**D**) The effects of *Ntf3* overexpression from adult supporting cells on ABR thresholds (**C**) and P1 amplitudes (**D**) after noise exposure; *n* = 5–9. *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA. Key in **D** applies to **C**–**D**. (**E**) Representative confocal images of IHC synapses from 32 kHz region of *Ntf3^{stop}* and *Ntf3^{stop}:Slc1a3/CreER^{T}* cochleae immunolabeled for CtBP2 and GluA2. The samples were collected from mice without AT/Tmx (Pre-AT/Tmx) or 14 days after AT/Tmx (AT/Tmx + 14 days). Scale bar = 10 μm. (**F**) *Ntf3* overexpression after acoustic trauma promotes regeneration of IHC ribbon synapses; *n* = 5–6. **p < 0.01, ***p < 0.001 by two-way ANOVA. Gray shading indicates the noise exposure spectrum.

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