ELSEVIER

Neurobiology of Aging xx (2010) xxx

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Age-related neuronal loss in the cochlea is not delayed by synaptic modulation

David Jin[a], Kevin K. Ohlemiller[a], Debin Lei[a], Elizabeth Dong[a], Lorna Role[b], David K. Ryugo[c], Jianxin Bao[a,d,e,*]

[a] *Department of Otolaryngology, Washington University, St. Louis, MO, United States*
[b] *Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, NY, United States*
[c] *Center for Hearing and Balance, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD, 21205, USA*
[d] *Center for Aging, Washington University, St. Louis, MO, United States*
[e] *The Division of Biology & Biomedical Science and Neuroscience Program, Washington University, St. Louis, MO, United States*

Received 15 November 2009; received in revised form 25 April 2010; accepted 12 May 2010

Abstract

Age-related synaptic change is associated with the functional decline of the nervous system. It is unknown whether this synaptic change is the cause or the consequence of neuronal cell loss. We have addressed this question by examining mice genetically engineered to over- or underexpress *neuregulin-1* (*NRG1*), a direct modulator of synaptic transmission. Transgenic mice overexpressing *NRG1* in spiral ganglion neurons (SGNs) showed improvements in hearing thresholds, whereas *NRG1* −/+ mice show a complementary worsening of thresholds. However, no significant change in age-related loss of SGNs in either *NRG1* −/+ mice or mice overexpressing *NRG1* was observed, while a negative association between *NRG1* expression level and survival of inner hair cells during aging was observed. Subsequent studies provided evidence that modulating *NRG1* levels changes synaptic transmission between SGNs and hair cells. One of the most dramatic examples of this was the reversal of lower hearing thresholds by "turning-off" *NRG1* overexpression. These data demonstrate for the first time that synaptic modulation is unable to prevent age-related neuronal loss in the cochlea.

Keywords: Aging; Presbycusis; *Neuregulin-1*; Spiral ganglion neuron; Cochlea

1. Introduction

Functional decline of the nervous system is a cardinal feature of normal aging (Yankner et al., 2008). In the central nervous system (CNS), current views suggest that loss of neuronal connections rather than loss of neurons may be the major cause of age-related functional decline (Morrison and Hof, 2007; Rapp and Gallagher, 1996; Rattner and Nathans, 2006; Scheff and Price, 2003). In the peripheral nervous system (PNS), age-related loss of both synapses and neurons contribute to this functional decline (Coggan et al., 2004; Bao et al., 2005; Ohlemiller, 2006; Thrasivoulou et al., 2006). It is unclear, however, whether age-related synaptic change is the cause or simply an associated manifestation of neuronal loss in the PNS. One way to address this issue directly is to examine whether modulations of synaptic transmission at the adult stage can change age-related neuronal loss.

Neuregulin-1 (*NRG1*) is known to be important for synaptic transmission (Corfas et al., 2004; Mei and Xiong, 2008; Talmage and Role, 2004). The *NRG1* gene encodes over 15 transmembrane and secreted isoforms (Falls, 2003; Fischbach and Rosen, 1997). Based on different amino-termini, *NRG1* isoforms are classified into 3 types: *type-I NRG1* has an immunoglobulin-like (Ig-like) domain, followed by a region of high glycosylation; *type-II* has a kringle-like domain plus an Ig-like domain; *type-III* has a

* Corresponding author at: Department of Otolaryngology, Center for Aging, Washington University in St Louis, Box 8115, 660 South Euclid Avenue, St. Louis, MO 63110, United States. Tel.: +1 314 747 7199; fax: +1 314 747 7230.
E-mail address: jbao@wustl.edu (J. Bao).

0197-4580/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2010.05.011

cysteine-rich domain. Most isoforms are transmembrane proteins, which contain an epidermal growth factor (EGF)-like extracellular domain, a transmembrane region, and an intracellular cytoplasmic domain. Extensive work has shown that NRG1 plays a critical role for synaptic transmission by both its forward and backward signaling pathways (for review see Mei and Xiong, 2008). NRG1 forward signaling pathways, via binding of its extracellular domains to erbB receptors, are able to regulate the expression of synaptic proteins such as neurotransmitter receptors and ion channels (Li et al., 2007; Liu et al., 2001; Okada and Corfas, 2004; Okazi et al., 1997; Zhong et al., 2008). The forward signaling pathways also contribute to the development and maturation of glial cells (Adlkofer and Lai, 2000; Birchmeier and Nave, 2008; Taveggia et al., 2005). NRG1 backward signaling pathways, via nuclear translocation of its cytoplasmic domain, are able to upregulate apoptotic and synaptic gene expression (Bao et al., 2003, 2004). In the cochlea, *type-III NRG1* is highly expressed in postsynaptic spiral ganglion neurons (SGNs). Its erbB receptors are present in presynaptic hair cells, Schwann glial cells, and supporting cells of the organ of Corti (Bao et al., 2003; Hume et al., 2003; Morley, 1998; Stankovic et al., 2004; Zhang et al., 2002). Therefore, NRG1 signaling is critical for synaptic transmission between hair cells and SGNs.

Hearing loss (presbycusis) is the third most prevalent complaint of the elderly (Gates and Mills, 2005; Ohlemiller and Frisina, 2008). In human presbycusis, a pattern of progressive hearing loss, typically starting at the high frequencies, corresponds to a loss of hair cells and SGNs in the basal region of the cochlea. This pattern is observed in C57BL/6J inbred mice, a well-studied animal model for presbycusis (Ohlemiller, 2006). In this model, age-related functional changes in synaptic transmission between inner hair cells and SGNs can be indirectly assessed using the amplitude of Wave I of the auditory brainstem response (ABR) (Melcher and Kiang, 1996). To test whether improvement of synaptic transmission between hair cells and SGNs in adult mice could delay age-related losses of hair cells and SGNs, we established a conditional tissue-specific transgenic model to express *NRG1* in mouse SGNs after 2 months of age, a time after which the development of the auditory system is complete (Rubel and Fritzsch, 2002). This approach is based on the tetracycline-regulated system used successfully for the conditional expression of a variety of genes in transgenic mice (Mansuy et al., 1998; Mayford et al., 1996; Yamamoto et al., 2000). Regulation of the system is achieved through the tetracycline-regulated transactivator (tTA), an artificial fusion protein between the tet-repressor binding domain and a VP16 activation domain. This protein binds specifically to the tetO operator and induces transcription from an adjacent CMV minimal promoter. The combination of both the tTA and tetO elements allows for the continuous expression of a given transgene after induction. Tetracycline or its analog, doxycycline (dox), can bind to tTA and prevent its binding to tetO, thereby inhibiting transcription (Gossen and Bujard, 1992). Tissue-specific expression is achieved by controlling the expression of *tTA* under a tissue-specific promoter (Mayford et al., 1996). The advantage of this system is the ability to inhibit transgenic expression at any desired time point, which allows us to directly test our hypothesis without causing developmental complications due to transgene expression.

2. Methods

2.1. Generating NRG1 transgenic mice and animal care

The *NRG1* transgenic lines were generated by cloning a mouse DNA fragment of whole *NRG1 III-β1a* fused with *GFP* into a pBI-3 plasmid. The new plasmid contained a bidirectional *tetO* sequence flanked by *CMV* minimal promoters with *lacZ* reporter sequences on 1 side and *NRG1* fused with a 4 × lysine sequence followed with *GFP* and valine at the end on the opposite side. The valine was added at the end of GFP because it affects the maturation (including routing and cleavage) of type 1 membrane proteins (Bosenberg et al., 1992; Briley et al., 1997). Based on our previous studies (Bao et al., 2003, 2004), the cellular distribution and cleavage of this transgene are similar to endogenous *NRG1*. The whole 7.1 kilobase (kb) PstII-SalI fragment, containing both transgenes, was cut out, gene-cleaned, and microinjected into single-cell CBAxC57BL/6J embryos. The founder was crossed with wild-type CBAxC57BL/6J females. Southern analysis of the founders was used to determine the copy numbers and the integration sites. Positive F1 lines were crossed to the CamKIIα-tTA B line to generate the complete conditional transgenic mouse. Eight of these mice were examined and 2 expressing *NRG1* in SGNs were crossed back to C57BL/6J (10 times). Mice were housed 5 per cage with food and water available. They were maintained in a noise-controlled environment on a 12-hour light/dark cycle, with light onset at 6:00 AM. Mice on dox treatment were given 2 mg/mL dox in a 5% sucrose solution. Dox solution was maintained in dark bottles and changed once a week.

2.2. LacZ staining

The *LacZ* reporter gene in all 8 transgenic lines has the advantage of permitting highly sensitive visualization of β-galactosidase activity in all tissue expressing the transgene (a blue reaction product in the cell). After fixation in 4% paraformaldehyde, tissue was washed 3 times in phosphate-buffered saline (PBS) solution for 30 minutes each and then incubated with X-Gal staining (Melford Laboratories, Suffolk, UK) for 2–12 hours in the dark.

2.3. Real-time RT-PCR Assay for mRNA

Total ribonucleic acid (RNA) from individual mice (2 cochleae) at each age group was extracted using RNAqueous (Ambion, Austin, TX). To avoid any DNA contamination at

the final step of RNA extraction, DNase I, 1 μl was added to 49 μl elution buffer in the RNA extraction column. The solution was incubated at 37 °C for 15 minutes and then heated to 100 °C for 5 minutes to denature the DNase I. RNA was quantified with Ribogreen RNA quantification reagent (Molecular Probes, Eugene, OR). Prior to cDNA generation by reverse transcription, the quality of RNA was determined by examining ribosomal RNA integrity on a 3% denatured agarose gel. One fifth of the total RNA (50 μl) was reverse transcribed in 20-μL reaction mixtures using random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Standard concentration curves for *GAPDH* and tested genes were made using their respective cDNA plasmids at 5 dilutions. Primers used for real-time polymerase chain reaction (PCR) of these mouse genes were: *GAPDH*: 5-CCTGGCCAAGGTCATCCATGACAAC-3 and 5-TGTCATACCAGGAAATGAGCTTGAC-3; *EGFP*: 5-CGCACCATCTTCTTCAAGGACGAC-3 and 5-AACTCCAGCAGGACCATGTGATCG-3; *Type-III NRG1*: 5-TCTAGTAAGCCTCTGCCTCTGCAT-3 and 5-GCGGTGGAGTGGAGTGTAAGGGA-3; *Type-II NRG1*: 5-GAAGAAGGACTCGCTACTCACC-3 and 5-GGCTGACCTCCTTCTTGAGG-3; *Type-I NRG1*: 5-ATGTCTGAGCGCAAAGAAGGCAGAGGCAA-3 and 5-CTGTATCTTGACGTTTTGTGGTTTATT-3; *ErbB2*: 5-TCTGCCTGACATCCACAGTG-3 and 5-CAGGGATCTCCCGAGCTGGG-3; *ErbB3*: 5-CTTACGGGACACAATGCTGA-3 and 5-GGCAAACTTCCCATCGTAGA-3; *ErbB4*: 5-TGAACAATGTGATGGCAGGT-3 and 5-TGAAGTTCATGCAGGCAAAG-3; *PSD-95*: 5-AGACTCGGTTCTGAGCTATG-3 and 5-TCTTTGGTAGGCCCAAGGAT-3; *SNAP-25*: 5-CCTGGGGCAATAATCAGGATGGAG-3 and 5-CGTTGGTTGGCTTCATCAATTCTGG-3.

The expression change of each gene was measured using the LightCycler system 1.5 (Roche, D-68298 Mannheim, Germany). PCR protocols of the LightCycler System for *GAPDH* and each tested gene were optimized with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). One tenth of each 20 μL cDNA was added to a 20 μL PCR reaction mixture containing 1 × PCR buffer, 0.5 μM of each primer, and Master Mix from the kit. The number of completed PCR cycles when the fluorescence intensity exceeded a predetermined threshold was measured automatically during PCR. The second derivative maximum method by the LightCycler Software 3.5.3 (Roche) was used to set this predetermined threshold for each sample, thus, potential human errors were avoided across different age groups. Quantification of the amount of template molecules was achieved by first establishing the standard curve for each gene (including *GAPDH*), then determining the concentration of each in the sample based on its standard curve. The difference in the initial amount of total RNA among the samples was normalized in every assay by dividing the concentration of each gene by the concentration of the house keeping gene, *GAPDH*. Besides using melting curve analysis to ensure the right PCR product, each PCR product was examined on a 3% agarose gel. The initial PCR products were cloned and sequenced to ensure correct identity.

2.3. Functional assays

Mice were anesthetized (80 mg/kg ketamine, 15 mg/kg xylazine, i.p.) and positioned dorsal side up in a custom head holder. Core temperature was maintained at 37 °C using a thermostatically controlled heating pad in conjunction with a rectal probe (Yellow Springs Instruments Model 73 A, Yellow Springs, OH). Platinum needle electrodes (Grass, West Warwick, RI) were inserted subcutaneously just behind the right ear (active), at the vertex (reference), and in the back (ground). Electrodes were led to a Grass P15 differential amplifier (0.1–10 kHz, ×100), to a custom broadband amplifier (0.1–10 kHz, ×1000), then digitized at 30 kHz using a Cambridge Electronic Design (Cambridge, UK) micro1401, in conjunction with SIGNAL and custom signal averaging software, operating on a 120 MHz Pentium PC. Sine wave stimuli generated by a Hewlett Packard 3325a digital oscillator were shaped by a custom electronic switch to 5.0 milliseconds total duration, including 1 ms rise/fall times. The stimuli were amplified by a Crown (Elkhart, IN) D150A power amplifier and led to an Alpine (Torrance, CA) SPS-OEOA coaxial speaker located 10 cm directly lateral to the right external auditory meatus. Stimuli were presented free field and calibrated using a B&K (Buffalo, NY) 4135 .25-inch microphone placed where the pinna would normally be. Tone burst stimuli at each frequency and level were presented 1000 times at 20/second. The minimum sound pressure level required for a response (short-latency negative wave) was determined at 5.0, 10.0, 14.2, 20.0, 28.3, 40.0, and 56.6 kHz, using a 5 dB minimum step size. To establish the input and output curves of ABR Wave I at 10 kHz, we determined the ABR threshold at 10 kHz for each animal (around 16 dB for 2 months old, and 41 dB for 12 months old), and then measured the amplitude of Wave I from peak to baseline. The sound level was increased in 5 dB steps and terminated at 101 dB.

2.4. Quantification of SGNs, missing IHCs, and OHCs

Mice were administered a lethal dose of sodium pentobarbital and when completely unresponsive, were perfused transcardially with cold 2% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered saline (PBS). Each cochlea was rapidly isolated, immersed in the same fixative, and the stapes immediately removed. Complete infiltration of the cochlea by fixative was ensured by making a small hole at the apex of the cochlear capsule and gently circulating the fixative over the cochlea using a transfer pipet. After overnight decalcification in sodium EDTA, cochleae were postfixed in buffered 1% osmium tetroxide, dehydrated in an ascending acetone series, and embedded in Epon. Cochleae were sectioned parallel to the modiolar axis at a thickness of 50 μm. A Nikon microscope equipped with

a motorized stage controlled by Stereo Investigator software was used for precise, well-defined movements along the x, y, and z 3-dimensional axes. High-resolution images and a thin focal plane were obtained using a 60× oil immersion objective lens from Nikon with a numerical aperture of 1.40. The optical fractionator method was used to sample the SGN number in a fraction of the spiral ganglion as defined by the optical dissector. A clear nucleolus, a large nucleus, and a clearly defined, oval body were used to distinguish SGNs from glial cells. SGN counts were made based on visualization of their nucleolus. For the counts of missing inner hair cells (IHCs) and outer hair cells (OHCs), the sections were reconstructed to include entire length of mouse cochlear basilar membrane with the Stereo Investigator software by focusing on the pillar heads between IHCs and OHCs. We focused the counts at 40%–70% from the cochlear apex using the 60× oil immersion objective lens. The criterion for identification of missing hair cells was their replacement with a phalangeal scar. The percentage loss of IHCs and OHCs was then divided into 3 segments that represented 10% of the organ of Corti length. All procedures followed the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee.

3. Results

3.1. Characterization of transgenic mice

NRG1 transgenic mice conditionally expressing mouse type-*III NRG1* in SGNs were established based on the pBI-3 vector, which contains a bidirectional tetO responsive promoter. This promoter controls the expression of the transgene *NRG1-EGFP* in 1 direction and the reporter gene *LacZ* in another direction (Fig. 1A). The major advantage of using this bidirectional tetO promoter is the relative ease in monitoring the expression of the *LacZ* reporter gene by simple but sensitive histological staining for the enzymatic activity of β-galactosidase. Eight transgenic lines were obtained and crossed to 1 transgenic line with the *tTA* transgene under the control of a calcium/calmodulin kinase II promoter (CamKIIα-tTA). In 2 transgenic lines, expression of the marker gene, *LacZ*, was found in SGNs of the cochlea (Fig. 1B), and this expression could be controlled by the administration of doxycycline (dox) in the drinking water (Fig. 1C). Because the expression pattern of both lines in the cochlea was identical, we focused on the 1 with greater SGN LacZ staining for further studies.

The level of transgene expression was quantified by real-time RT-PCR in the cochlea. As expected, the *EGFP* expression level was significantly higher in 4-month-old animals without dox treatment (turned-on). The average concentration ratio between *EGFP* and *GAPDH* was 1.30 ± 0.4 (10^{-4}) for the "turned-on" group, and 0.28 ± 0.2 (10^{-4}) for the noninduced control group with dox in their drinking water from pregnancy to 4 months of age ("turned-off"). Therefore, there was about 4.6-fold induction in our trans-

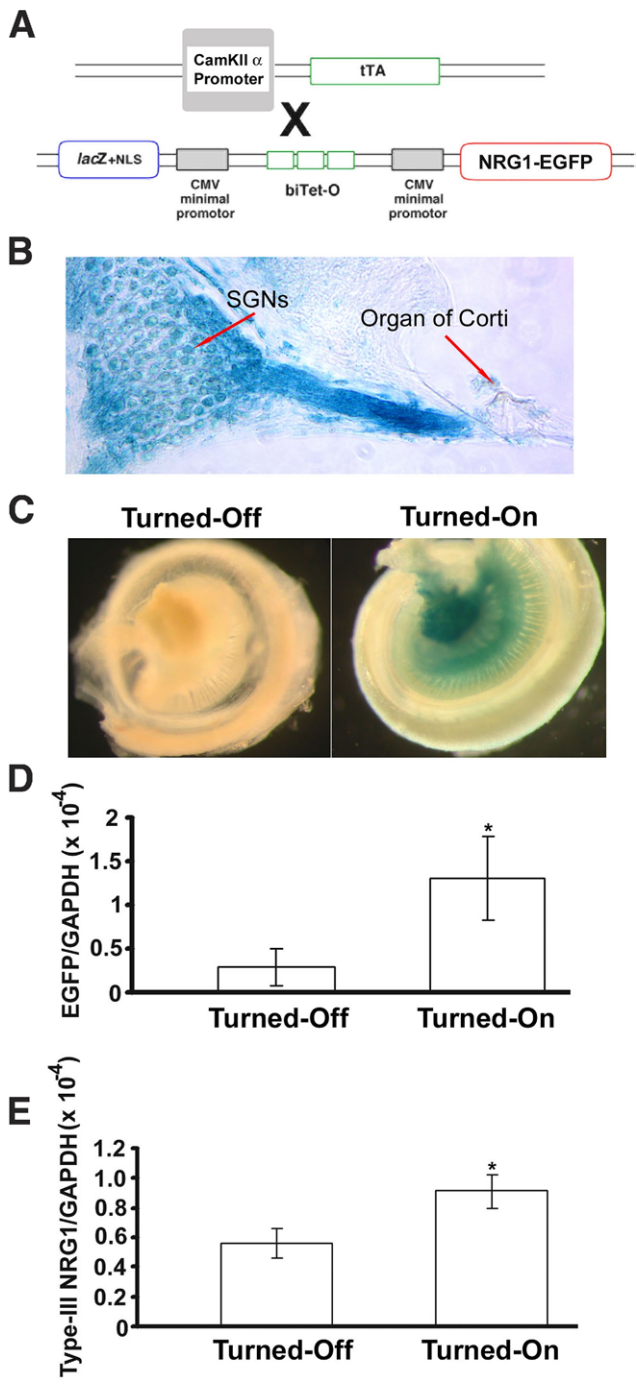

Fig. 1. Validation of *NRG1* conditionally tissue-specific transgenic mice. (A) Schematic diagram of the conditionally tissue-specific transgenic model, which resulted from a crossing of 2 transgenic mouse lines. One line had the *tTA* expression under the CamKIIα promoter, and another line we made contained the biTet-O promoter controlling the expression of both the reporter gene *LacZ* and the transgene *NRG1-EGFP*. (B) The LacZ staining showed that spiral ganglion neurons (SGNs) were the only cell type expressing the *LacZ*. (C) The SGN-specific *LacZ* expression could be turned on or off by doxycycline (dox). (D) and (E) Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) quantifications of the transgene expression (with *EGFP* primers) or the total expression of *NRG1* in the cochlea ($n = 4$, t test, $p < 0.05$).

genic model (Fig. 1D). Similarly, a moderate but significant increase of total *type-III NRG1* was found in the "turned-on" group (Fig. 1E). To examine whether overexpression of *type-III NRG1* altered expression of other *NRG1* isoforms or their receptors, we examined the expression level of *NRG1 type-I* and *type-II*, *erbB2*, *erbB3*, and *erbB4* in both "turned-on" and their siblings without the transgene construct (WT). There was no significant difference between the "turned-on" and control groups for the expression of these 5 genes, while there was a significant difference for *type-III NRG1* between the same 2 groups (Fig. 2). The expression level of *type-III NRG1* was 0.43 ± 0.1 (10^{-4}) for the control, 0.56 ± 0.1 (10^{-4}) for the "turned-off", and 0.91 ± 0.1 (10^{-4}) for the "turned-on".

3.2. Nrg1 levels and age-related hearing loss

To test whether *NRG1* overexpression in SGNs could delay age-related hearing loss, we compared the hearing threshold between the control and *NRG-1* overexpression mice under the same C57/6J genetic background at 12 months of age. Dox was withdrawn at 2 months of age to induce *NRG1* overexpression in the "turned-on" group. ABR measurements showed a moderate but significant reduction of hearing loss for the "turned-on" group (Fig. 3A). In order to further characterize the relationship between the level of *NRG1* and hearing loss, we examined *type-III NRG1* −/+ mice under the 129/Sv genetic background. *NRG1* expression level in the cochleae of heterozygous mice at 4 months of age was significantly lower than in controls (Fig. 3B). The ratio between *type-III NRG1* and *GAPDH* was about 1.4×10^{-4} for *type-III NRG1* −/+ mice and 2.6×10^{-4} for their wild-type sibling controls. We next compared hearing thresholds between the heterozygous mice and their wild-type sibling controls. Opposite to the findings in *NRG1* overexpression mice, at 12 months of age, *NRG1* −/+ mice showed significantly worse hearing (Fig. 3C).

To examine how *NRG1* levels influence age-related hearing loss, we examined the total number of SGNs in the same animals, and also quantified outer hair cell (OHC) and inner hair cell (IHC) loss in the region extending 40%–70% from the cochlear apex (subserving ~ 10–25 kHz; Ou et al., 2000). This region was selected for analysis because it was the site associated with the clearest preservation of hearing in *NRG1* transgenic mice and clearest exacerbation of hearing loss in *NRG1* −/+ mice (Fig. 3A and 3C). Surprisingly, no differences were found in age-related loss of SGNs between the control and *NRG1* overexpression mice (Fig. 4A; left). For missing OHCs (Fig. 4B; left), 2-way analysis of variance (ANOVA) analysis showed no differences in the mean values among genotypes ($p = 0.412$), locations ($p = 0.125$), or between genotypes and locations ($p = 0.494$). For missing IHCs (Fig. 4C; left), mice overexpressing *NRG1* even showed significantly more missing cells ($p = 0.006$) while no differences were observed in locations ($p = 0.252$) or between genotypes and locations ($p = 0.854$). Thus,

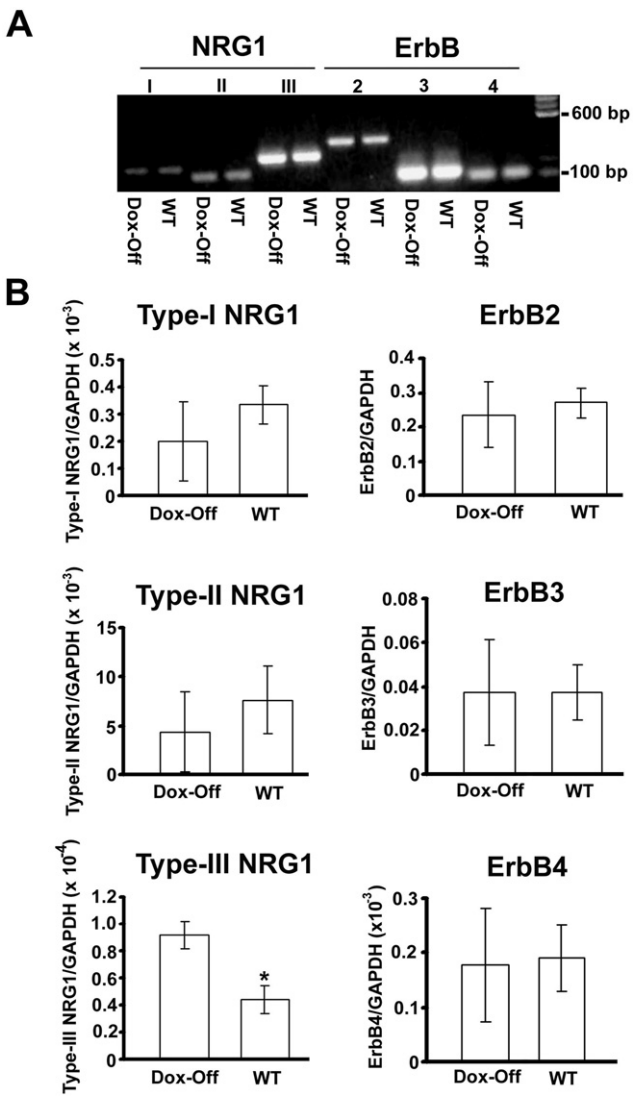

Fig. 2. No changes in the expression of *NRG1* isoforms or their receptors after *type-III NRG1* overexpression. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of type-*I*, *-II*, *-III NRG1*, and *ErbB 2*, *3*, *4* in cochlea from mice overexpressing *type-III NRG1* or their sibling mice without *NRG1* transgene at 4 months of age. No dramatic difference between the control and *NRG1* overexpressing mice was observed. (B) Real-time RT-PCR quantification of the same groups of gene expression in the cochlea. No significant difference was found between the control and *NRG1* overexpression mice for NRG1 isoforms or their receptors except *type-III NRG1* ($n = 4$, *t* test).

NRG1 overexpression during aging failed to protect SGNs and was toxic to IHCs.

Similarly, *NRG1* −/+ mice showed no significant difference versus controls in the survival of SGNs (Fig. 4A; right). For missing OHCs (Fig. 4B; right), 2-way ANOVA showed no differences in the mean values among genotypes ($p = 0.439$), locations ($p = 0.349$), or between genotypes and locations ($p = 0.709$). For missing IHCs (Fig. 4C; right), no difference was found among genotypes ($p = 0.761$) or locations ($p = 0.745$). There was a difference

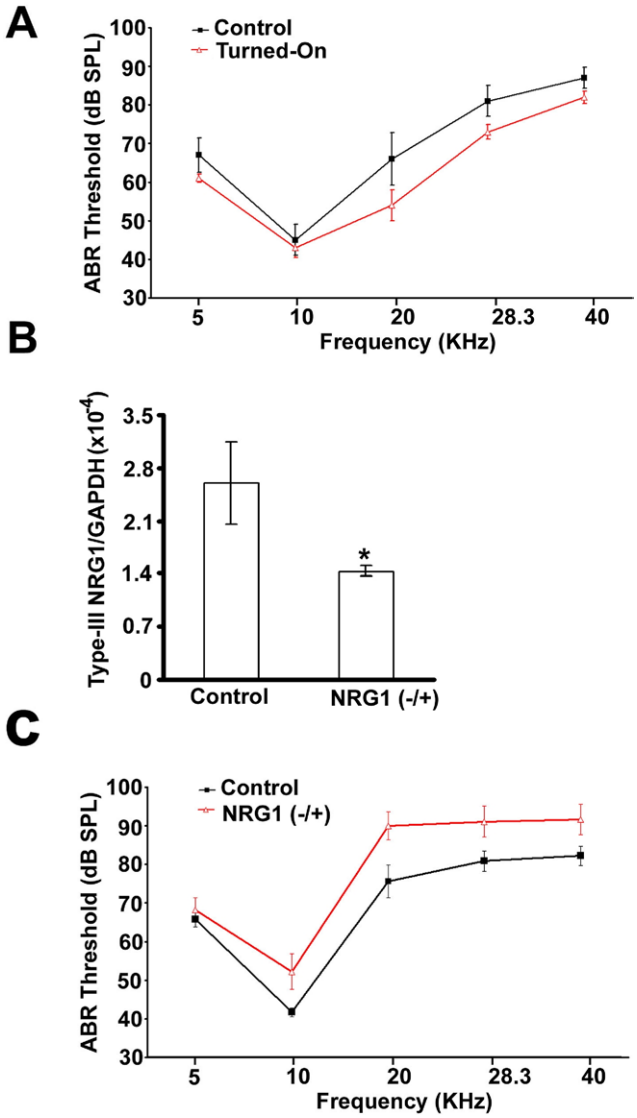

Fig. 3. The *NRG1* level modulates auditory brainstem response (ABR) thresholds. (A) ABR thresholds (mean ± SD) for mice overexpressing *NRG1* (red) and their sibling mice without *NRG1* transgene (control; black) at 12 months of age ($n = 5$ for each group, 2 females and 3 males; analysis of variance [ANOVA], $p < 0.05$). (B) Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) quantification of *type-III NRG1* in mouse cochlea for the control and *NRG1* −/+ mice at 4 months old ($n = 4$; *t* test, $p < 0.05$). (C) ABR thresholds (mean ± SD) for the control ($n = 8$) and *NRG1* −/+ mice ($n = 9$) at 12 months old (ANOVA, $p < 0.05$).

between genotypes and locations ($p = 0.011$): the number of missing IHCs was less in *NRG1* −/+ mice. In summary, neither improvements in hearing thresholds in the presence of *NRG1* overexpression, nor worsening of hearing in the presence of reduced *NRG1* levels (*NRG1* −/+ mice), were associated with changes in SGN survival. However, the survival of IHCs was negatively correlated with the expression level of *NRG1*. To explain this enigmatic finding, we reasoned that *NRG1* overexpression might increase only synaptic transmission between hair cells and SGNs, which reduces ABR thresholds. Thus, we further studied possible links between *NRG1* levels and synaptic changes in the cochlea during aging.

3.3. Age-related changes in the expression of synaptic genes

We first examined whether the improved hearing thresholds after *NRG1* overexpression were due to changes in the expression of synaptic proteins. Using real-time RT-PCR, we measured the expression level of genes encoding 2 synaptic proteins: (1) the postsynaptic marker postsynaptic density protein-95 (PSD-95); and (2) the presynaptic marker synaptosomal-associated protein-25 (SNAP-25). At 4 months of age, the level of PSD-95 in the cochlea was not significantly different between these 2 groups of mice (Fig. 5A). Similar to findings in the hippocampus (Nyffeler et al., 2007), the PSD-95 level dramatically increases in the cochlea in the 12-month-old control group. Interestingly, this age-related increase of PSD-95 was reduced after *NRG1* overexpression. A similar observation was also made for the expression of *SNAP-25*: a significant increase of expression during aging in the control group, but not in the "turned-on" group (Fig. 5B). These data clearly showed that age-related high expression levels for these 2 genes were reduced after *NRG1* overexpression. However, decreased expression of 2 important synaptic genes could not explain an improved hearing threshold after *NRG1* overexpression.

3.4. Detailed temporal analysis of hearing thresholds during aging

We further analyzed the temporal changes of hearing thresholds in the control and "turned-on" groups at 2, 4, 8, and 10 months of age (Fig. 6). Because the transgenic *NRG1* gene was "turned on" at 2 months of age, there was no difference in the hearing thresholds between control and "turned-on" groups at this age. At 4 months of age, however, a difference in the hearing thresholds at high frequency regions (10–40 kHz) was observed. A similar difference was observed at 8 and 10 months. The difference in the hearing threshold between these 2 groups was relatively constant from 4 to 10 months of age at about 5 dB on average.

Similar to the function of *NRG1* at the neuromuscular junction (Fischbach and Rosen, 1997; Rimer, 2007), we reasoned that if the *NRG1* level modulates the synaptic transmission between hair cells and SGNs, the amplitude of the ABR Wave I would be larger in *NRG1* overexpression mice and smaller in the *NRG1* −/+ mice. Because the elevated hearing thresholds during aging could make this method less sensitive, we focused on the 10 kHz cochlear region, which is the most sensitive hearing frequency region for mice. The amplitude of a 10 kHz tone was increased in 5 dB increments to establish the input-output relation for each mouse. At 2 months of age, there was no difference between control and *NRG1* overexpressing mice (Fig. 7A). At 12 months of age, average response amplitudes were

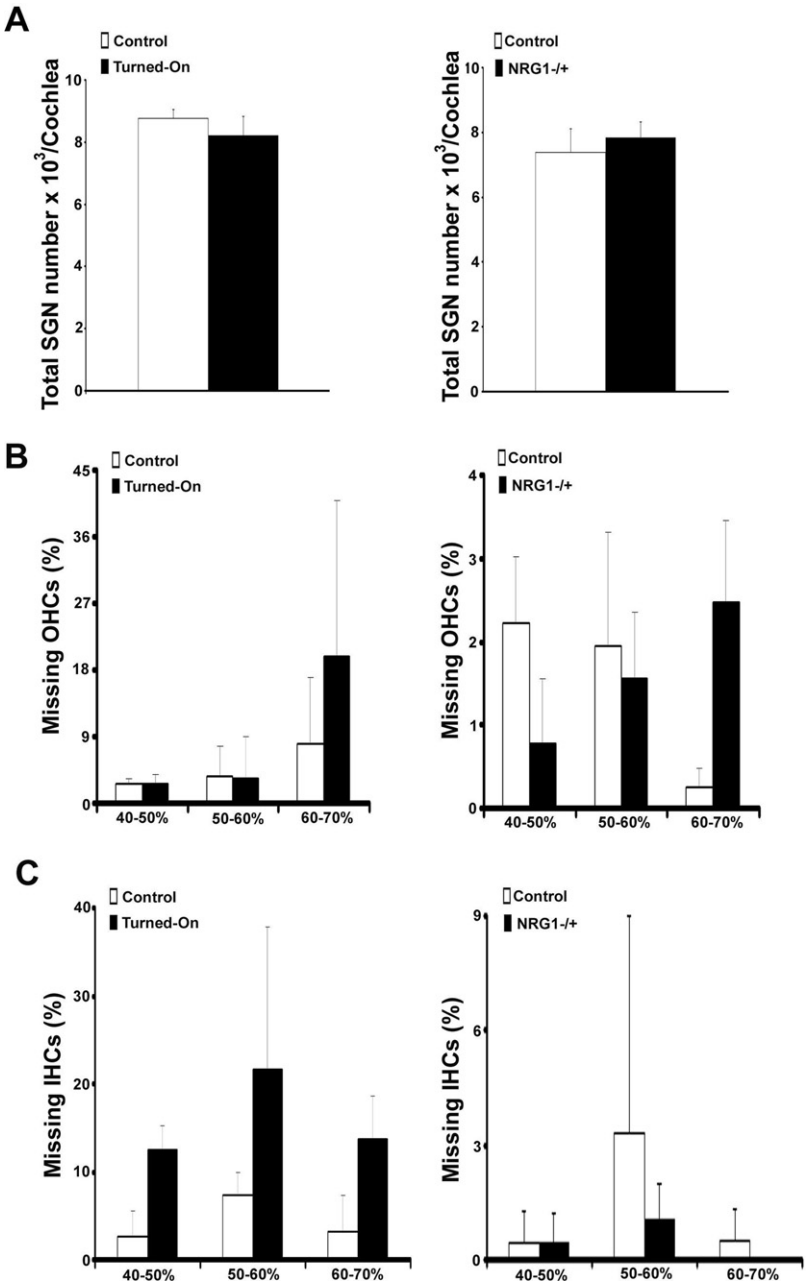

Fig. 4. Quantitative comparisons of spiral ganglion neurons (SGNs), outer hair cells (OHCs), and inner hair cells (IHCs) between the control and *NRG1* overexpression mice, or the control and *NRG1* −/+ mice. (A) Quantitative comparison of total SGN number among the same 4 groups of animals tested in Fig. 3. No significant difference was detected between the control and transgenic mice at 12 months old (*t* test). (B) Quantitative comparison of missing OHCs at the 40%–70% region from the apex (2-way analysis of variance [ANOVA]). (C) Quantitative comparison of missing IHCs in the same area (2-way ANOVA).

significantly higher in overexpressing mice (Fig. 7B), and significantly lower above 81 dB in *NRG1* −/+ mice (Fig. 7C). Although ABR thresholds (Fig. 6) and input-output curves for Wave I (Fig. 7) only indirectly reflect synaptic transmission between hair cells and SGNs, they support the argument that *NRG1* levels modulate the synaptic connection between hair cells and SGNs. Thus, the improvement of hearing thresholds in 12-month-old mice overexpressing *NRG1* could be due to an enhancement of synaptic transmission between SGNs and IHCs, which could even compensate an age-related loss of IHCs.

3.5. NRG1 overexpression temporarily changes hearing thresholds

If the above suggestions were correct, a transient improvement of ABR hearing thresholds during aging would be predicted to disappear as soon as *NRG1* overexpression

was "turned-off". Our conditional tissue-specific transgenic mice were ideal for this purpose (Yamamoto et al., 2000). In the control group, transgenic *NRG1* expression was "turned-off" throughout life by continuous administration of dox. In another group, *NRG1* overexpression was "turned-on" at 2 months of age, which led to an improvement of ABR thresholds at high frequencies at 4 months old. *NRG1* overexpression was then "turned-off" by application of dox (turned-on-off). After 2 weeks of dox administration, the improvement of ABR thresholds dramatically disappeared (Fig. 8). The data were consistent with that *NRG1* improved the hearing thresholds by enhancing synaptic transmission between hair cells and SGNs similar to its function at the neuromuscular junction (Fischbach and Rosen, 1997; Rimer, 2007).

4. Discussion

Our results showed that *NRG1* could regulate hearing thresholds in mice. Conditional *NRG1* overexpression in SGNs improved hearing thresholds, whereas hearing thresh-

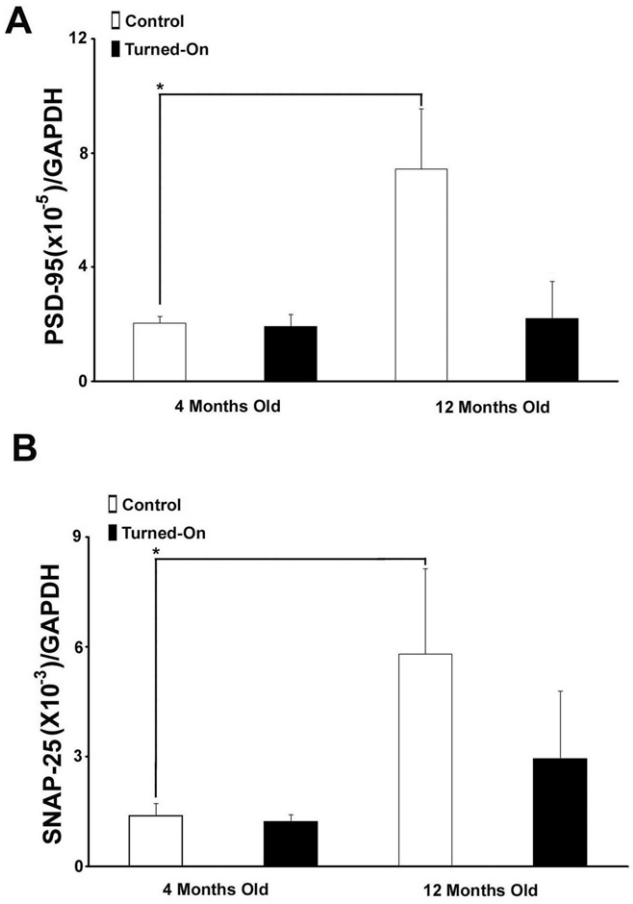

Fig. 5. Quantitative comparisons of the expression of 2 synaptic genes during aging between the control and *NRG1* overexpression mice. Age-related upregulation of postsynaptic density protein-95 (PSD-95) (A) and synaptosomal-associated protein-25 (SNAP-25) (B) was found in the cochlea, and this significant upregulation was diminished for these 2 genes after *NRG1* overexpression ($n = 4$, t test).

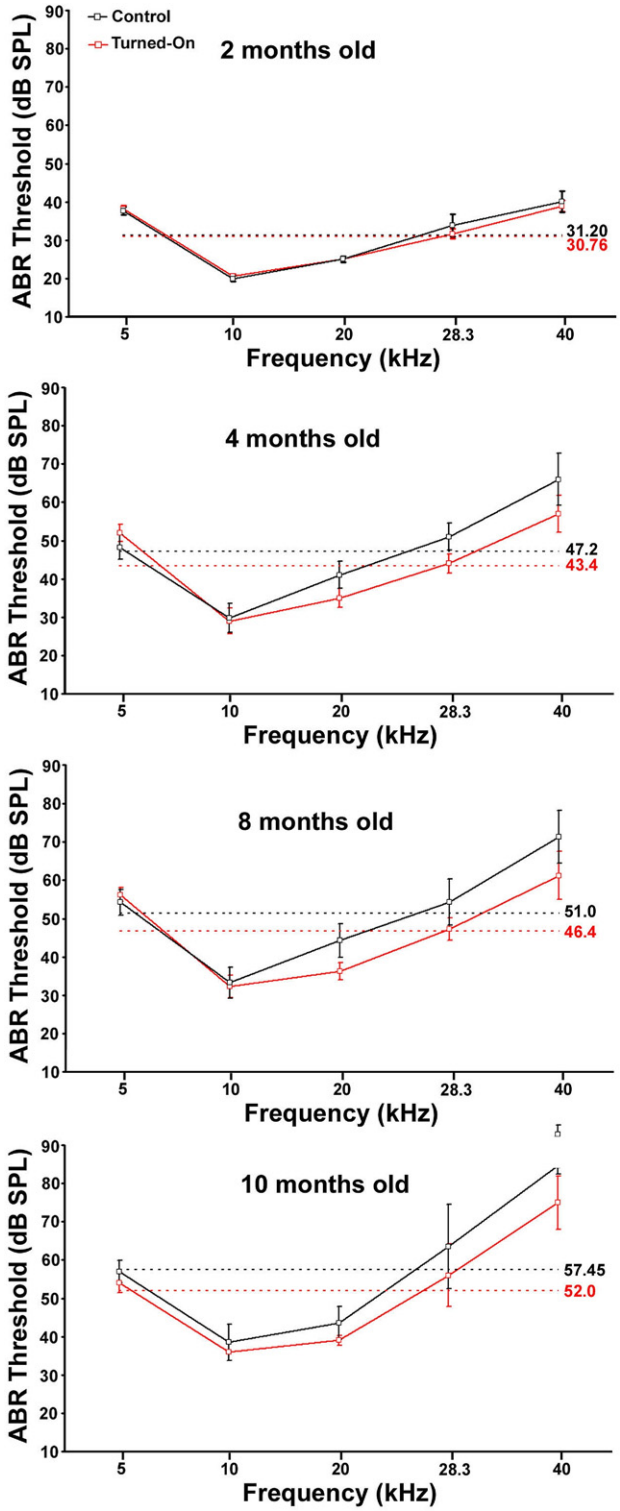

Fig. 6. Comparisons of auditory brainstem response (ABR) hearing thresholds between the control and *NRG1* overexpression mice during aging. ABR threshold shifts (mean ± SD) for both the control and *NRG1* overexpression mice were measured at 5, 10, 20, 28.3, and 40 kHz ($n = 9$ for each group, 5 females and 4 males). The dotted line for each group represents the average threshold from these 5 measurements. The average thresholds increased during for both group, but the differences in average threshold between the 2 groups remained almost the same across ages, around 5 dB.

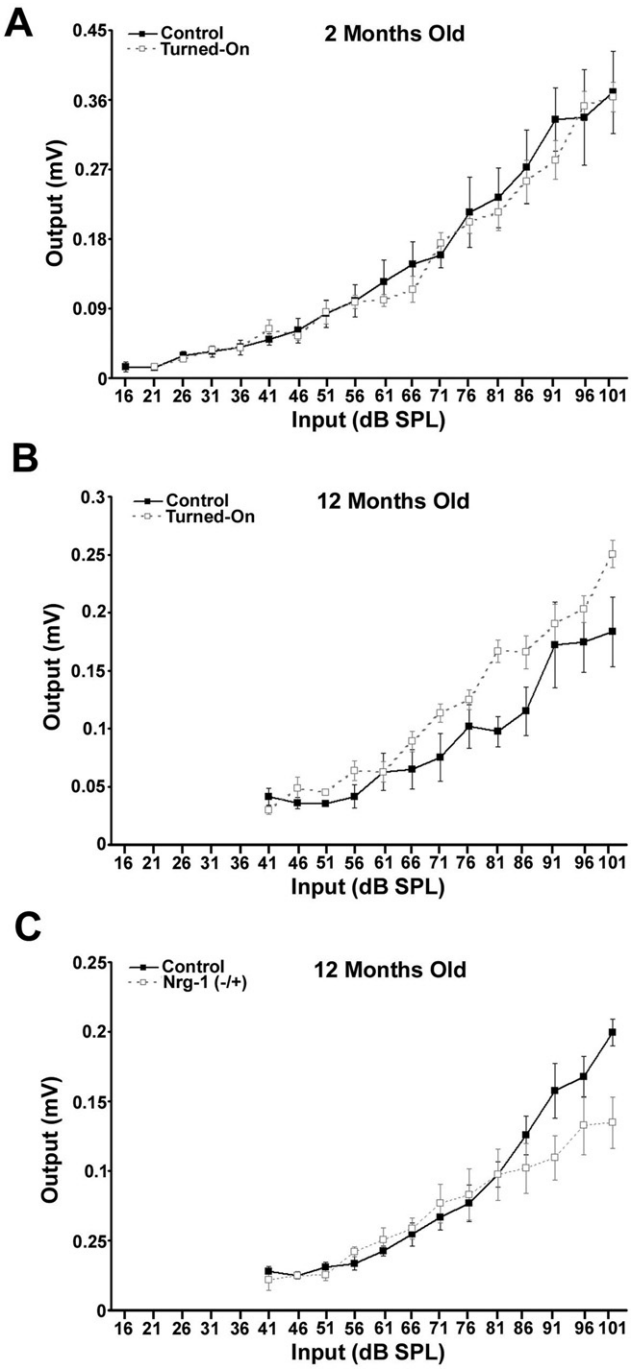

Fig. 7. Comparisons of the input-output curves of Wave I between the control and *NRG1* transgenic mice. (A) At 2 months old, there was no difference between these 2 groups under C57/6J genetic background in their input-out curves ($n = 5$; 2-way analysis of variance [ANOVA]). (B) At 12 months old, the input-output curve was much higher for *NRG1* overexpression group. Please note that the amplitude at the same input intensity was much lower compared with the amplitude from 2-month-old mice ($n = 5$; 2-way ANOVA, $p < 0.001$). (C) At 12 months old, the input-out curve was lower at the high intensity range (over 81 dB) for the *NRG1* −/+ mice, which expressed a lower amount of *NRG1* ($n = 8$; 2-way ANOVA, $p < 0.001$).

olds worsened in heterozygous mice (*NRG1* −/+) having a subnormal amount of *NRG1*. The decrease of hearing thresholds after *NRG1* overexpression did not result from preservation of SGNs or hair cells during aging, but was associated with increased amplitude of ABR Wave I. Finally, improvement of hearing thresholds in *NRG1* overexpression mice was reversed when *NRG1* overexpression was "turned-off". These observations suggest that the improvement in hearing sensitivity is due to the enhancement of synaptic transmission between SGNs and hair cells by *NRG1*. Thus, our novel finding suggests that enhancing synaptic transmission during aging does not delay age-related neuronal loss in the cochlea.

In the cochlea, previous studies have clearly demonstrated degeneration of SGN synaptic terminals and loss of SGNs and hair cells during aging (Stamataki et al., 2006; White et al., 2000; Zimmermann et al., 1995). It was unclear whether synaptic loss in the PNS was the cause or the consequence of age-related neuronal loss. We originally thought that loss of SGN synaptic terminals contributed to loss of SGNs and hair cells during aging. The conclusion based on current data suggested that high *NRG1* levels could improve synaptic transmission in the cochlea without protecting SGNs (even harmful to IHCs) in vivo during aging. However, the amount of *NRG1* expression is determined only at the mRNA level, which may not reflect its expression at the protein level. In addition, it is still impossible to directly measure synaptic transmission between hair cells and SGNs in vivo during aging; we did not have the direct evidence to prove that *NRG1* could enhance synaptic transmission between single IHC and SGN. Nevertheless, the conclusion has been strongly supported by the following observations: (1) the difference in hearing threshold at different ages (from 2 to 12 months old) showed a consistent

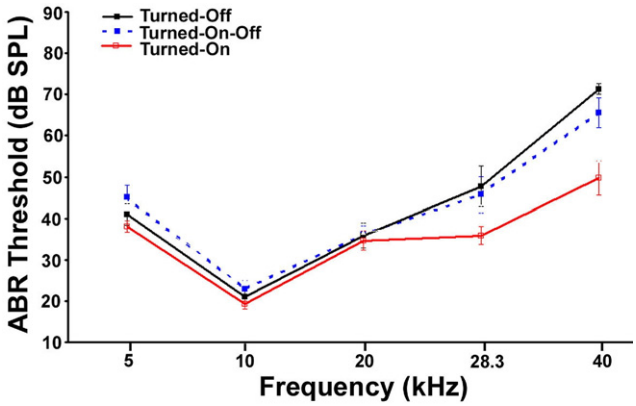

Fig. 8. Reversibility of improved auditory brainstem response (ABR) thresholds. ABR thresholds (mean ± SD) were measured at 4 months old for the group with *NRG1* overexpression always "turned off" ($n = 7$; solid black), or the group with *NRG1* overexpression "turned on" at 2 months old ($n = 9$; solid gray). ABR thresholds (mean ± SD) were measured again for the late group after *NRG1* overexpression was subsequently "turned off" for 2 weeks.

4–5 dB improvement for mice moderately overexpressing *NRG1* compared with control mice; (2) the amplitude of Wave I at 10 kHz was related to the *NRG1* level: a significant increase after *NRG1* overexpression and a significant decrease in *NRG1* −/+ mice respectively; (3) the improvement in hearing sensitivity offered by *NRG1* overexpression was transient and disappeared as soon as it was "turned-off" by administration of doxycycline; and (4) there was an age-related change in the expression of synaptic proteins after cochlear *NRG1* overexpression. The last piece of the argument needed further discussion because it was counter-intuitive.

Synaptic loss is a hallmark of normal aging; however, studies have demonstrated an age-related increase in total synaptic contact area (Scheff and Price, 2003). Age-induced increases in protein expression of PSD-95, the gluR1 subunit of AMPA channels (Nyffeler et al., 2007), synapsin I, and the alpha subunit of the type II voltage-gated sodium channel (Burger et al., 2007) have been also found. It has been suggested that the increase in total synaptic contact area, along with increased synaptic protein expression, act as compensatory mechanisms to alleviate the age-related loss of synaptic transmissions (Nyffeler et al., 2007; Scheff and Price, 2003). In our study, *NRG1* was expressed only by SGNs in the cochlea (Bao et al., 2003; Morley, 1998). Any changes in synaptic marker expression due to *NRG1* overexpression came mainly from the synapse between hair cells and SGNs. Because increasing *NRG1* expression had no effect in the expression of genes encoding both pre- and post-synaptic proteins at 4 months of age, and we observed a less pronounced increase in expression of the same synaptic markers compared with controls at 12 months age, the data suggested a preservation of synaptic transmission between SGNs and hair cells after *NRG1* overexpression. In addition, we observed a negative association between the expression level of *NRG1* and IHC survival. In the central nervous system (CNS), alteration of *NRG1* signaling can modulate glutamatergic synaptic transmission through α-amino 3-hydroxy-5-methyl-4 isoazolepropionic acid (AMPA) receptors (for reviews, see Corfas et al., 2004; Mei and Xiong, 2008). In the cochlea, AMPA receptors are present both presynaptically (GluR4) and postsynaptically (GluR2 and 3; Chen et al., 2007; Luo et al., 1995; Matsubara et al., 1996). Therefore, the negative association between the expression level of *NRG1* and IHC survival may be due to glutamate-induced excitotoxicity via AMPA receptors whose expression levels could be mediated by *NRG1* signaling during aging. Further studies are needed to determine this possibility.

Extensive synaptic and neuronal loss contributes to the pathology of age-related neurodegenerative diseases such as Alzheimer's disease (Morrison and Hof, 2007; Scheff and Price, 2003; Smith et al., 2000; Yankner et al., 2008). This raises a similar but clinically important question of whether synaptic loss in this case is the cause or the result of neuronal loss. Our data suggest a third possibility, a parallel independent biological process for age-related loss of neurons and synapses. Because neuronal aging is the common predisposing factor for neurodegenerative diseases, this possibility is worth exploring. In addition, our data suggest that the level of *NRG1* can modulate synaptic transmission in the cochlea, similar to the neuromuscular junction. Modulating *NRG1* function might be used clinically for tinnitus, by transiently diminishing synaptic transmission in the cochlea.

Disclosure statement

There are no actual or potential conflicts of interest for this work.

All procedures followed the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee.

Acknowledgements

Special thanks to Barbara Bohne for her critical reading of the manuscript. This research has been funded in part by grants from the National Institute of HealthR01AG024250, R21DC010489, DC004395, P30DC004665, and T32DC000022-22.

References

Adlkofer, K., Lai, C., 2000. Role of neuregulins in glial cell development. Glia 29, 104–111.

Bao, J., Lei, D., Du, Y., Ohlemiller, K.K., Beaudet, A.L., Role, L.W., 2005. Requirement of nicotinic acetylcholine receptor subunit beta2 in the maintenance of spiral ganglion neurons during aging. J. Neurosci. 25, 3041–3045.

Bao, J., Lin, H., Ouyang, Y., Lei, D., Osman, A., Kim, T.W., Mei, L., Dai, P., Ohlemiller, K.K., Ambron, R.T., 2004. Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. Nat. Neurosci. 7, 1250–1258.

Bao, J., Wolpowitz, D., Role, L.W., Talmage, D.A., 2003. Back signaling by the Nrg-one intracellular domain. J. Cell Biol. 161, 1133–1141.

Birchmeier, C., Nave, K.A., 2008. Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. Glia 56, 1491–1497.

Bosenberg, M.W., Pandiella, A., Massagué, J., 1992. The cytoplasmic carboxy-terminal amino acid specifies cleavage of membrane TGFalpha into soluble growth factor. Cell 71, 1157–1165.

Briley, G.P., Hissong, M.A., Chiu, M.L., Lee, D.C., 1997. The carboxyl-terminal valine residues of proTGF alpha are required for its efficient maturation and intracellular routing. Mol. Biol. Cell 8, 1619–1631.

Burger, C., López, M.C., Feller, J.A., Baker, H.V., Muzyczka, N., Mandel, R.J., 2007. Changes in transcription within the CA1 field of the hippocampus are associated with age-related spatial learning impairments. Neurobiol. Learn. Mem. 87, 21–41.

Chen, Z., Kujawa, S.G., Sewell, W.F., 2007. Auditory sensitivity regulation via rapid changes in expression of surface AMPA receptors. Nat. Neurosci. 10, 1238–1240.

Coggan, J.S., Grutzendler, J., Bishop, D.L., Cook, M.R., Gan, W., Heym, J., Lichtman, J.W., 2004. Age-associated synapse elimination in mouse parasympathetic ganglia. J. Neurobiol. 60, 214–226.

Corfas, G., Roy, K., Buxbaum, J.D., 2004. Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. Nat. Neurosci. 7, 575–580.

Falls, D.L., 2003. Neuregulins: functions, forms, and signaling strategies. Exp. Cell Res. 284, 14–30.

Fischbach, G.D., Rosen, K.M., 1997. ARIA: a neuromuscular junction neuregulin. Annu. Rev. Neurosci. 20, 429–458.

Gates, G.A., Mills, J.H., 2005. Presbycusis. Lancet 366, 1111–1120.

Gossen, M., Bujard, H., 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551.

Hume, C.R., Kirkegaard, M., Oesterle, E.C., 2003. ErbB expression: the mouse inner ear and maturation of the mitogenic response to heregulin. J. Assoc. Res. Otolaryngol. 4, 422–443.

Li, B., Woo, R., Mei, L., Manilow, R., 2007. The neuregulin-one receptor erbB4 controls glutamatergic synapse maturation and plasticity. Neuron 54, 583–597.

Liu, Y., Ford, B., Mann, M.A., Fischbach, G.D., 2001. Neuregulins increase alpha7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus. J. Neurosci. 21, 5660–5669.

Luo, L., Brumm, D., Ryan, A.F., 1995. Distribution of non-NMDA glutamate receptor mRNAs in the developing rat cochlea. J. Comp. Neurol. 361, 372–382.

Mansuy, I.M., Winder, D.G., Moallem, T.M., Osman, M., Mayford, M., Hawkins, R.D., Kandel, E.R., 1998. Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 21, 257–265.

Matsubara, A., Laake, J.H., Davanger, S., Usami, S., Ottersen, O.P., 1996. Organization of AMPA receptor subunits at a glutamate synapse: a quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. J. Neurosci. 16, 4457–4467.

Mayford, M., Bach, M.E., Huang, Y.Y., Wang, L., Hawkins, R.D., Kandel, E.R., 1996. Control of memory formation through regulated expression of a CaMKII transgene. Science 274, 1678–1683.

Mei, L., Xiong, W.C., 2008. Neuregulin 1 in neuronal development, synaptic plasticity and schizophrenia. Nat. Rev. Neurosci. 9, 437–452.

Melcher, J.R., Kiang, N.Y., 1996. Generators of the brainstem auditory evoked potential in cat. III: Identified cell populations. Hear. Res. 93, 52–71.

Morley, B.J., 1998. ARIA is heavily expressed in rat peripheral auditory and vestibular ganglia. Brain Res. Mol. Brain Res. 54, 170–174.

Morrison, J.H., Hof, P.R., 2007. Life and death of neurons in the aging cerebral cortex. Int. Rev. Neurobiol. 81, 41–57.

Nyffeler, M., Zhang, W.N., Feldon, J., Knuesel, I., 2007. Differential expression of PSD proteins in age-related spatial learning impairments. Neurobiol. Aging 28, 143–155.

Ohlemiller, K.K., 2006. Contributions of mouse models to understanding of age- and noise-related hearing loss. Brain Res. 1091, 89–102.

Ohlemiller, K.K., Frisina, R.D., 2008. Clinical characterization of age-related hearing loss and its neural and molecular bases, in: Schacht, J., Popper, A., Fay, R. (Eds.), Auditory Trauma, Protection and Treatment. Springer-Verlag, New York; pp 45–194.

Okada, M., Corfas, G., 2004. Neuregulin1 downregulates post-synaptic GABAA receptors at the hippocampal inhibitory synapse. Hippocampus 14, 337–344.

Okazi, M., Sasner, M., Yano, R., Lu, H.S., Buonanno, A., 1997. Neuregulin-beta induces expression of NMDA-receptor subunit. Nature 390, 691–694.

Ou, H.C., Harding, G.W., Bohne, B.A., 2000. An anatomically based frequency-place map for the mouse cochlea. Hear. Res. 145, 123–129.

Rapp, P.R., Gallagher, M., 1996. Preserved neuron number in the hippocampus of aged rats with spatial learning deficits. Proc. Natl. Acad. Sci. U. S. A. 93, 9926–9930.

Rattner, A., Nathans, J., 2006. Macular degeneration: recent advances and therapeutic opportunities. Nat. Rev. Neurosci. 7, 860–872.

Rimer, M., 2007. Neuregulins at the neuromuscular synapse: past, present, and future. J. Neurosci. Res. 85, 1827–1833.

Rubel, E.W., Fritzsch, B., 2002. Auditory system development: primary auditory neurons and their targets. Annu. Rev. Neurosci. 25, 51–101.

Scheff, S.W., Price, D.A., 2003. Synaptic pathology in Alzheimer's disease: a review of ultrastructural findings. Neurobiol. Aging 24, 1029–1046.

Smith, T.D., Adams, M.M., Gallagher, M., Morrison, J.H., Rapp, P.R., 2000. Circuit- specific alterations in synaptophysin immunoreactivity predict spatial learning impairments in aged rats. J. Neurosci. 20, 6587–6593.

Stamataki, S., Francis, H.W., Lehar, M., May, B.J., Ryugo, D.K., 2006. Synaptic alterations at inner hair cells precede spiral ganglion cell loss in aging C57BL/6J mice. Hear. Res. 221, 104–118.

Stankovic, K., Rio, C., Xia, A., Sugawara, M., Adams, J.C., Liberman, M.C., Corfas, G., 2004. Survival of adult spiral ganglion neurons requires erbB receptor signaling in the inner ear. J. Neurosci. 24, 8651–8661.

Talmage, D.A., Role, L.W., 2004. Multiple personalities of neuregulin gene family members. J. Comp. Neurol. 472, 134–139.

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R.M., Loeb, J.A., Shrager, P., Chao, M.V., Falls, D.L., Role, L., Salzer, J.L., 2005. Neuregulin-one type III determines the ensheathment fate of axons. Neuron 47, 681–694.

Thrasivoulou, C., Soubeyre, V., Ridha, H., Giuliani, D., Giaroni, C., Michael, G.J., Saffrey, M.J., Cowen, T., 2006. Reactive oxygen species, dietary restriction and neurotrophic factors in age-related loss of myenteric neurons. Aging Cell 5, 247–257.

White, J.A., Burgess, B.J., Hall, R.D., Nadol, J.B., 2000. Pattern of degeneration of the spiral ganglion cell and its processes in the C57BL/6J mouse. Hear. Res. 141, 2–18.

Yankner, B.A., Lu, T., Loerch, P., 2008. The aging brain. Annu. Rev. Pathologe 3, 41–66.

Yamamoto, A., Lucas, J.J., Hen, R., 2000. Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 101, 57–66.

Zhang, M., Ding, D., Salvi, R., 2002. Expression of heregulin and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium. Hear. Res. 169, 56–68.

Zhong, C., Du, C., Hancock, M., Mertz, M., Talmage, D.A., Role, L.W., 2008. Presynaptic type III neuregulin 1 is required for sustained enhancement of hippocampal transmission by nicotine and for axonal targeting of alpha7 nicotinic acetylcholine receptors. J. Neurosci. 28, 9111–9116.

Zimmermann, C.E., Burgess, B.J., Nadol, J.B., 1995. Patterns of degeneration in the human cochlear nerve. Hear. Res. 90, 192–201.