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Age-related Hearing Loss: GABA, Nicotinic Acetylcholine and NMDA Receptor Expression Changes in Spiral Ganglion Neurons of the Mouse

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Abstract

Age-related hearing loss – presbycusis – is the number one communication disorder and most prevalent neurodegenerative condition of our aged population. Although speech understanding in background noise is quite difficult for those with presbycusis, there are currently no biomedical treatments to prevent, delay or reverse this condition. A better understanding of the cochlear mechanisms underlying presbycusis will help lead to future treatments. Objectives of the present study were to investigate gamma-amino butyric acid A ($GABA_A$) receptor subunit α1, nicotinic acetylcholine (nACh) receptor subunit β2, and N-methyl-D-aspartate (NMDA) receptor subunit NR1 mRNA and protein expression changes in spiral ganglion neurons of the CBA/CaJ mouse cochlea, that occur in age-related hearing loss, utilizing quantitative immunohistochemistry and semi-quantitative RT-PCR techniques. We found that auditory brainstem response (ABR) thresholds shifted over 40 dB from 3–48 kHz in old mice compared to young adults. DPOAE thresholds also shifted over 40 dB from 6–49 kHz in old mice, and their amplitudes were significantly decreased or absent in the same frequency range. Spiral ganglion neuron (SGN) density decreased with age in basal, middle and apical turns, and SGN density of the basal turn declined the most. A positive correlation was observed between SGN density and ABR wave 1 amplitude. mRNA and protein expression of $GABA_AR$ α1 and AChR β2 decreased with age in SGNs in the old mouse cochlea. mRNA and protein expression of NMDAR NR1 *increased* with age in SGNs of the old mice. These findings demonstrate that there are functionally-relevant age-related changes of $GABA_AR$, nAChR, NMDAR expression in CBA mouse SGNs reflecting their degeneration, which may be related to functional changes in cochlear synaptic transmission with age, suggesting biological mechanisms for peripheral age-related hearing loss.

Keywords

Aging; Hearing loss; Cochlea; Spiral Ganglion Neurons; Gene expression; Protein expression

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Introduction

Age-related hearing loss is a disorder caused by mixed pathology including both genetic and environmental factors. Schuknecht classified presbycusis into four subtypes: (1) sensory (loss of hair cells), neural (loss of spiral ganglion neurons, [SGNs]), metabolic (atrophy of the stria vascularis and decline of the endolymphatic potential) and mechanical (cochlear anatomical changes, including alterations of the basilar membrane) (Schuknecht. 1964; Schuknecht et al. 1993). "Sensorineural" presbycusis refers to the high-frequency-hearing impairment resulting from loss of hair cells and degeneration of SGNs. It is a major contributor to age-related hearing loss. In addition, SGN degeneration during aging contributes to functional declines in the output of the peripheral auditory system that can induce plasticity changes in the central auditory system. Functionally, SGNs connect hair cells with central auditory system neurons in the cochlear nucleus of the brainstem; the first neurons in the central auditory pathway. Thus, to study the etiologies of SGNs degeneration during aging paves a way to prevent or slow down the progression of age-related hearing loss as well as providing insights for other types of hearing impairment.

Gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the brain and auditory system. The $GABA_A$ receptor ($GABA_AR$) is a key inhibitory receptor; playing a critical role in regulating neuronal excitability and information processing in the nervous system. $GABA_AR$ is a pentameric structure formed by its multi-gene family, and there are at least 19 different members of the $GABA_AR$ family, including α1-6, β1-3, γ1-3, ρ1-3, δ1, ε1, θ1, and π1. The majority of $GABA_ARs$ consist of 2α, 2β, and 1γ subunit. In the adult brain, the α1β2γ2 subtype is the major subtype accounting for 43% of all $GABA_ARs$ (McKernan et al. 1996). The majority of $GABA_ARs$ are widely expressed in the auditory system (Campos et al. 2001; Yomamoto et al. 2002) and as one of its functions, it plays an important cochlear protective role via the auditory efferent system (Arnold et al. 1998; Murashita et al. 2007). Our previous studies (Tang et al. 2010) showed that the majority of the $GABA_AR$ subunits were expressed in the cochlea, and there were age-related changes of GABARs in the mouse cochlea (D'Souza et al. 2008), and central auditory system (Raza et al. 1994). However, age-related changes of $GABA_AR$ in the inner ear have not been investigated and it may be that age-related hearing loss is partially due to $GABA_AR$ changes with age in both peripheral and central auditory systems.

The N-methyl-D-aspartate receptor (NMDAR) is a glutamate-gated ion channel ubiquitously distributed throughout the brain; and it is fundamental for excitatory neurotransmission. NMDARs are composed of heteromeric complexes of NR1 and NR2 subunits. A functional NMDAR appears to consist of two classes of subunits NR1 and NR2 (AD). NR1 is the fundamental subunit necessary for the NMDAR complex (Watanabe et al. 1992; Wada et al. 2004). NMDA receptors are expressed at hair cell/afferent nerve synapses, playing a crucial role in neurotransmission and synaptic plasticity and excitotoxicity. Age-related changes of NMDARs have been reported in the central auditory system (Osumi et al. 2012). Our previous study showed that all the subunits were expressed in the cochlea (Tang et al. 2010). Investigation of NMDARs expression changes with age will help our understanding of mechanisms of presbycusis.

Acetylcholine (ACh) is a main transmitter released by the medial olivocochlear efferent fibers (Fuchs et al. 1992). nAChRs are mainly located at the synapse between efferent fibers and outer hair cells (OHC). It is now believed that the hair cell cholinergic receptor that mediates synaptic transmission between efferent olivocochlear fibers and hair cells, is formed by both α9 and α10 subunits. The activation of the hair cell nAChR leads to an increase in intracellular Ca+-activated K+ (SK2) channels thus leading to hyperpolarization

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of hair cells and reduction of electromotility (Dulon et al. 1998; Oliver et al. 2000). Located on the peripheral projections of SGNs are α2, α4-7, and β2-3 nAChR subunits (Housley et al. 1991), and it has been shown that the nAChR β2 subunit is required for the maintenance of SGNs during aging (Bao et al. 2005). Also in the central nervous system, loss of β2 results in region specific alterations in cortex, including neocortical hypotrophy, loss of hippocampal pyramidal neurons, and astro- and micro-gliosis. Spatial learning is significantly impaired as well (Zoli et al. 1999). nAChR β2 is critical in the maintenance of normal neuronal function. However, the exact functions of the nAChRs in SGN synapses remain elusive.

The auditory system of the CBA/CaJ strain has important similarities to the human system, particularly at peripheral and brainstem levels; therefore it has proven to be a very useful model for studying the biological mechanisms of presbycusis. In this present study we report on the detection and age changes of $GABA_A$R α1, nACh β1 and NMDAR NR1 subunits; using protein and mRNA expression level methodologies for young adult (2–3 month) and old age (24–32 month) CBA/CaJ mice to increase our structure-function understanding of pharmacological and electrophysiological characteristics of these receptors.

Materials and methods

Animals

CBA/CaJ mice were bred in-house under 12h:12h light-dark cycle. Young adult (2–3 months, n=12) and old (24–32 months, n=12) were used for the auditory brainstem response (ABR) physiology experiments. Subsets of these physiologically-characterized young adult (Y) and old (O) mice were utilized in the other multidisciplinary experiments as follows. Distortion product otoacoustic emissions (DPOAE): Y, n=6; O, n=6. For the immunocytochemistry procedures: ACh, Y, n=6; O, n=5. NMDA, Y, n=8; O, n=7. GABA, Y, n=4; O, n=4. The opposite-side cochleae were used for the PCR experiments: Y, n=6, and O, n=6. All the mice were examined under the microscope for their external ear canal and tympanic membrane; mice with any symptoms of ear infection were excluded from the study. All the animal procedures were approved by the University of South Florida Committee on Animal Resources and are consistent with US Federal and NIH guidelines. Auditory brainstem response (ABR) and distortion-product emissions recordings were tested prior to sacrificing, and the procedures were the same as our previous reports (Zettel et al. 2007; D'Souza et al. 2008; Frisina et al. 2011).

Auditory brainstem responses (ABR) recordings

Mice were anesthetized with a mixture of ketamine/xylazine (120 and 10mg/kg body weight, respectively, intraperitoneal injection). ABRs were measured in response to tone pips of 3, 6, 12, 16, 20, 24, 32, 48 kHz and wide band noise presented at a rate of 11 bursts/s. Briefly, ABR recordings were obtained with a Tucker-Davis Technologies (TDT) System III workstation running BioSigRP in a sound booth (IAC). ABRs were measured with subcutaneous platinum needle electrodes placed at the vertex (non-inverting input), right mastoid prominence (inverted input), and back (indifferent site). Tone pips of 5 msec duration and 0.5 msec rise-fall time (phase alternating 90°) were utilized. Signals were calibrated in a cavity whose volume approximated the volume of the mouse ear canal using a 1/8″ B&K 4138 microphone. Threshold was defined as the lowest intensity which elicited a clearly replicable response. Normal body temperature was maintained at 37°C with a servo heating pad.

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Distortion-product otoacoustic emissions (DPOAEs)

Distortion-product otoacoustic emissions were obtained following ABR audiometry, under anesthesia (ketamine/xylazine, 120 and 10 mg/kg, respectively) a TDT combination microphone/speaker system was placed in the test ear near the tympanic membrane with a small, custom acoustic coupler. Acoustic stimuli were delivered and DPOAE waveforms were recorded using BioSig-RP software and TDT System III hardware. The digitally synthesized stimulus (200 kHz sample rate) consisted of two primary pure-tone frequencies (f1 and f2) differing by a factor of 1.25, at 65 and 50dB SPL, respectively. A fast Fourier transform was performed on the resultant waveform recorded from the insert microphone, and the spectral magnitude of the two primaries, 2f1-f2 distortion product, and the noise floor were determined. The procedure was repeated for f1 frequencies ranging from 5.6 to 44.8 kHz (eight frequencies per octave) to adequately assess the neuroethologically relevant functional range of mouse hearing.

Immunocytochemistry

After anesthetization with ketamine (120 mg/kg) and xylazine (10 mg/kg) for 30 minutes, the mice were sacrificed, and the cochleae were dissected and fixed in 4% paraformaldehyde in PBS (PH=7.4) overnight at 4°C, decalcification in 10% EDTA in PBS for a week at 4°C, then cryoprotected overnight at 4°C in cryoprotectant solution (30% sucrose & 10% polyvinyl-pyrrolidone in 0.1M PB and ethylene glycol). The cochleae were then embedded in degassed OCT overnight at 4°C, oriented into a cryomold with OCT degassed for 1 hour, then frozen at −80°C. Cryosectioning was performed at 5 μm per section (Thermo Scientific Inc., Waltham, MA). The slides were dried, washed with PBS for 3×5 min, then blocked in 0.1% Triton X-100,10% goat serum in PBS for 1 hour at room temperature (20–25°C), then incubated with the primary antibody: $GABA_AR\alpha1$ (1:100, rabbit polyclonal, ab33299 Abcam, Cambridge, MA), NMDARNR1 (1:100, rabbit polyclonal, ab17345, Abcam) or nAChRβ2 (1:100, rabbit polyclonal, sc-11372, Santa Cruz Biotechnology, Inc., Dallas, TX) overnight at 4°C. Anti-rabbit secondary antibody (HRP, rabbit, Cell Signaling, Danvers, MA) was used to visualize the primary antibodies; then the slides were stained with DAB (Vector Labs, Burlingame, CA) and mounted with cover glasses using Toluene Solution (Fisher Scientific, Waltham, MA). Images were captured using a digital camera (EOS Rebel T3i, Canon) interfaced with the light microscope (Leica DMR). The quantitative expression of staining for each receptor was calculated by obtaining the mean values of densitometry from 80–100 positively labeled cells from basal, middle and apical turns of the cochlea from young adult and old mice (N=6 per group).

RNA extraction and RT-PCR detection

One cochlea from each subject was used for immunocytochemistry (above) and the other for gene expression: RT-PCR. The temporal bones were dissected under a microscope and under sterile conditions; the bony cover of the cochlea and the spiral ligament was removed, total RNA was extracted from the modiolus using the SV Total RNA Isolation System (Promega, Madison, WI). 10 ng of total RNA was reverse transcribed to complementary DNA and PCR was performed according to instructions from the Enhanced Avian HS RT-PCR 100 Kit (HSR-T20, Sigma, St. Louis, MO). All mRNA sequences were from Genbank. The primers were designed using Primer Premier 5.0 software (Premier, Palo Alto, CA) Forward and reverse primer sequences, from the 5′ to 3′ direction were as follows: β-actin: sense 5′-GCTCTGGCTCCTAGCACCAT-3′, anti-sense 5′-GCCGGACTCATCGTACTCCT-3′, $GABA_AR\alpha1$: sense 5′-AATGGGCGGATTGGTGTC-3′, antisense 5′-TCATCTTGGGAGGGCTGT-3′; nAChRβ2 sense 5′-CTGTGGACGGTGTACGCTT-3′, antisense 5′-TCAGAGGGGAGGGAGAATG-3′; NMDARNR1 sense 5′-CAAGTGGGCATCTACAAT-3′, anti-sense 5′-TGACATACACGAAGGGTT-3′. PCR was

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initiated with an initial denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 8 minutes. Each set of reactions always included a no-sample negative control. We usually performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. The PCR amplified fragments were analyzed on a 1.5% agarose gel. All the samples were electrophoresed in the same agarose gel and expression levels were quantified by densitometry using Image J (National Institutes of Health, USA); Relative quantities of $GABA_AR$, nAChR and NMDAR mRNA were determined by $A_{GABAAR/nAChR/NMDAR}/A_{\beta\text{-actin}}$ ratios.

H&E staining and SGN counts

Sections were prepared for immunocytochemistry as mentioned above and stained with H&E (BBC Biochemical, Histo-Perfect, Mount Vernon, WA). The images of Rosenthal's canal were acquired at 1.25 × 10× magnification with the Leica bright-field microscope. Rosenthal's canal outline was traced to determine its total area, and all the SGNs (The cell bodies of the SGNs are found in the cochlear modiolus) from apical, middle and basal turns of young adult and old age mice (N=6 per subject group) were counted with the MetaMorph imaging system (Molecular Devices, Sunnyvale, CA). SGN density (number of SGNs/1000 μm^2) was calculated by dividing the number of SGNs by the area measured.

Statistical analysis

Statistical analysis was performed with Prism 6.0 software (GraphPad Software, La Jolla, CA). Repeated measures ANOVA and Student's t-test were used for group comparisons. Bonferroni post-hoc tests, corrected for multiple comparisons were used for ABR and DPOAE data. Linear regression was used for correlation analyses. A p value of less than 0.5 was regarded as statistically significant.

Results

ABR recordings

Overall, the ABR thresholds were significantly elevated in old age mice, as shown in Fig. 1 There were statistically significant differences at all frequencies ($F_{1,10}$=1218, $p<0.001$) between the young (2–3 months) and old (24–32 months) mice. The age-related threshold shift is over 40 dB from 3–48 kHz.

DPOAEs recordings

DPOAE amplitudes at 12 and 16 kHz were significantly lower in old mice as compared to young, and responses at the other frequencies were absent or in the system noise floor for the old mice ($F_{1,10}$=672.8, $p<0.001$; Fig. 2). DPOAEs thresholds were poorer, up to 40 dB for F2 frequencies of 6.7, 13.4, 17.9, 26.8, 33.5 and 49.2 kHz in old mice compared to young adults ($F_{1,10}$=1598, $p<0.001$; Fig. 3).

Gene expression changes with age

$GABA_AR$ α_1, nAChR β2 and NMDAR NR1 were detected in SGNs as presented in Figs. 4 and 5. mRNA and protein expression of $GABA_AR$ α_1 decreased with age in SGNs ($F_{1,10}$=89.432, $p<0.001$; Figs. 4A, 4B, 4G, 5A, 5B). mRNA and protein expression of nAChR β2 also decreased in all three turns in the cochlea with age ($F_{1,10}$=37.073, $p<0.001$; Figs. 4C, 4D, 4H, 5C, 5D). mRNA and protein expression of NMDAR NR1 *increased* in all three turns with age in SGNs ($F_{1,10}$=28.850, $p<0.001$; Figs. 4E, 4F, 4I, 5E, 5F).

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Spiral ganglion neuron loss with age

SGN density, as delineated by H&E staining, for basal, middle and apical turns all significantly decreased in the cochlea of old mice (Figs. 6B, 6D, 6F) when compared to young adult mice ($F_{1,10}$=34.264, p<0.001; Figs. 6A, 6C, 6E, 6G). Overall SGN density of the basal turn decreased the most ($F_{1,10}$=8.365, p=0.016; Figs. 6E and 6F).

Correlations between SGN density and ABR amplitude wave I

In old mice, ABR thresholds were found to be higher than 90 dB SPL, except for 12 and 16 kHz, which are generated in the middle turn of the cochlea (Muniak et al. 2013). We analyzed the correlations between SGN density and ABR wave I responses, and the results revealed a positive correlation between SGN density and ABR wave I amplitude (Fig. 7 and Fig. 8).

Discussion

Functional roles and age-linked changes of NMDAR, GABAAR and nAChR expression in the primary auditory pathway

NMDAR NR1 subunit expression does not change greatly during the development of the cochlear nuclei in chicken (Tang et al. 2004). NR1 expression increases in early development, then falls to and/or remains at an intermediate level into adulthood in rat (Akazawa et al. 1994) and mouse auditory brainstem (Tadros et al. 2007). However, NR1 expression declines in the inferior colliculus (IC) of aging animals, which indicates that NR1 may be involved in the pathogenesis of presbycusis (Osumi et al. 2012). NR1 expression in SGNs of the rat cochlea is *up-regulated* following kanamycin induced deafness (Hasegawa et al. 2000). Our study showed that NMDAR NR1 *increased* with age in mouse SGNs, which might be compensatory for decreased neuronal excitability in response to glutamate neurotransmitter system changes in the aging cochlea. Specifically, previous investigations have shown that the content of glutamate in the brain, including the central auditory system (Profant et al., 2013) and in the inner ear (Peng et al., 2013) primarily decrease with age. In contrast, the NMDAR NR1 *increases* seen in the present study, despite declining numbers of SGNs with age, may be geared towards maintaining neuronal excitability in cochlear afferent signal transduction during aging. Taken together with the previous studies of NMDAR in the auditory pathway, these results suggest that patterns of NMDAR expression changes in conjunction with age-related hearing loss may be different for the peripheral and central auditory systems.

Significant alterations of $GABA_A$ R subunits mRNA levels have been reported for the aging Fischer 344 rat IC (Milbrandt et al 1997). The γ1 subunit mRNA significantly *increased* in IC while the α1, β2 and γ2 subunits mRNA was observed to change very little with age. The alterations of the $GABA_A$ R subunits represent compensatory mechanisms in response to GABAergic system changes in the auditory brainstem reported by Caspary and colleagues (Raza et al. 1994; Milbrandt et al. 1997). It was also reported that $GABA_A$ R subunit expression showed age-related changes in rat auditory cortex (Cui et al. 1997; Xu et al. 2009; Caspary et al. 2012). In cochlea, GABA reduces NMDA-induced activity of afferent fibers (Bodarky et al. 2009; Moeller et al. 2010) . In addition, $GABA_A$R activation in the cochlea reduces probability of acoustic injury (Murashita et al. 2007). So $GABA_A$Rs serve a protective role in the peripheral auditory system. Also, GABAergic components of the olivocochlear system contribute to the long-term maintenance of hair cell and neuron survival in the inner ear (Maison et al. 2006). The present study discovered $GABA_A$R α1 subunit expression down-regulation with age, consistent with the idea that the normal protective actions of $GABA_A$Rs in the cochlea for hair cell and SGN survival are less

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effective with aging, thus contributing to the deleterious effects of age-related hearing loss in the inner ear.

ACh is a primary efferent neurotransmitter in the cochlea. Efferent protection is mediated via the α9 nAChRs in OHCs, which are responsible for cochlear efferent inhibition and provide some protection of the cochlea from noise over-exposure injury (Maison et al. 2000). SGNs express α2, α4-7, and β2-3 nAChR subunits. Mice lacking the nAChR subunit (β2−/−) have dramatic hearing loss and significant reduction in the number of SGNs, therefore, the β2 nAChR is required for maintenance of SGNs during aging (Bao et al. 2005). Consistent with this, the current study showed that nAChR β2 expression decreased in the presbycusis mouse model, likely contributing to functional hearing changes during aging.

There are synaptic interactions involving ACh, glutamate and GABA in the auditory system (Metherate et al. 1995). For instance, in auditory cortex, activation of NMDA receptors may reduce ACh release, and cholinergic agonists depress EPSPs mediated by glutamate; and ACh can also reduce monosynaptic GABAergic IPSPs. Spontaneous ACh release tonically depresses synaptic potentials mediated by glutamate and GABA. In sum, both the inhibitory and excitatory neurotransmitters and their receptors exist and interact in the auditory pathway for processing acoustic signals, and in the young adult there is an optimal balance between inhibitory and excitatory neurotransmitter systems that becomes disrupted with age. Changes in this delicate balance of inhibitory and excitatory neurotransmitters and their receptors during aging likely results in altered, degraded evoked responses to acoustical stimuli due to synaptic strength and inhibitory processing mis-matches in the auditory system (e.g., Kotak et al. 1997; Mossop et al. 2000).

Spiral ganglion neuron loss with age

The correlation between SGN density and ABR wave I amplitude underscores the possible interactions between SGN survival and ABR deficits and impaired cochlear function in the aging auditory system. Our study revealed that SGNs decrease with age in apical, middle and basal regions, with the basal area most affected. This is consistent with the tendency towards high frequency hearing loss in presbycusis and relations to hair cell pathologies in CBA mice (Spongr et al. 1997). Mechanistically, it is likely that SGNs rely on hair cell synaptic activity for trophic support. But alternatively, SGN loss can occur without damage or death of hair cells (Ryals et al. 1988; White et al. 2000; Linthicum et al. 2009). Indeed, in humans IHC loss does not always lead to SGN loss during aging (Jin et al. 2011). Additionally, mitochondrial damage caused by reactive oxygen species (ROS) can induce age-related SGN death via necrosis or apoptotic, programmed cell death (Yamasoba et al. 2007). Previous studies (Kujawa et al. 2006; Kujawa et al. 2009) have shown that early noise exposure can cause a reversible temporary hearing threshold increase with irreversible SGN loss, therefore early noise exposure is a critical environmental factor for SGN survival during age-related hearing loss. Genetic mutations can also predispose one to more rapid age-related cochlear dysfunction. For example, the glutamate receptor metabotropic 7 (GRM7) expressed in the hair cell/SGN synapse has been identified as one of the first presbycusis genes in humans (Newman et al. 2012). In sum, the present report underscores the importance of hair cell/SGN/efferent synapse neurotransmitter receptor changes with age, but additional research is required to better describe the molecular mechanisms of age-linked synaptic degradation and SGN death, and dysfunction during aging.

Conclusion

The present study demonstrated that there are age-related changes of $GABA_A$, nACh and NMDA receptor expression in CBA mouse primary auditory neurons that might contribute

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to neural degeneration and cochlear synaptic transmission as a function of age. Preventing or optimally modulating the age-related down-regulation of $GABA_AR$ α1, nAChR β2 and up-regulation of NMDAR NR1 expression levels before or during age-related hearing loss could contribute to preventing or minimizing presbycusis. In conclusion, the present report leads to more insights into the functions of $GABA_AR$ α1, nAChR β2 and NMDAR NR1 receptor protein regulation in the aging mammalian auditory system, paving the way for novel biomedical interventions to prevent or slow down the progression of age-related hearing loss as well as other types of hearing impairment.

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Highlights

1. Auditory brainstem response (ABR) thresholds shift over 40 dB from 3–48 kHz in old mice compared to young adults.
2. DPOAE thresholds shifted over 40 dB in old mice, and their amplitudes were decreased or absent in the same frequency range.
3. Spiral ganglion neuron (SGN) density decreased with age in the whole cochlea with the largest decline in the basal turn.
4. A positive correlation between SGN density and ABR wave I amplitude was discovered.
5. mRNA and protein expression of $GABA_AR$ α1 and nAChR β2 decreased while NMDAR NR1 increased with age in SGNs of the old mice.

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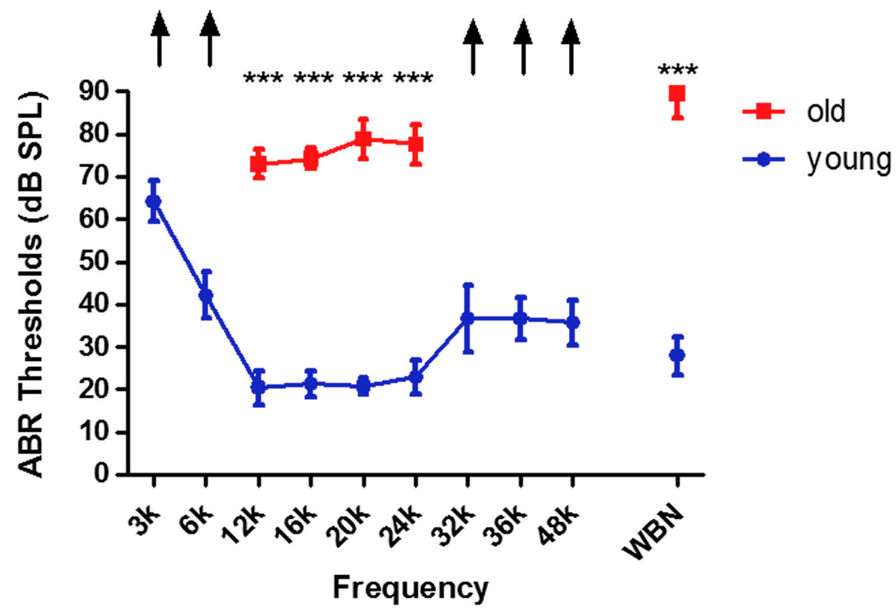

FIG. 1.
ABR thresholds of young adult and old age mice. ABR thresholds were significantly elevated for the old mice (*** $p<0.001$). Arrows indicate the thresholds exceeded the upper limits of the TDT ABR system. k = kHz. WNB- wideband noise stimulus.

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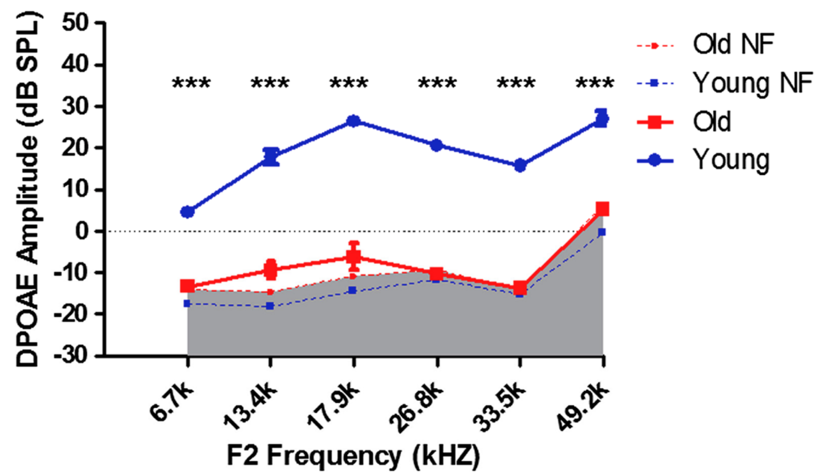

FIG. 2.

DPOAE amplitudes of young adult exceed those of and old mice, indicating significant loss of outer hair cell function with age. DPOAE (F1=65 dB SPL, F2=50 dB SPL f2/f1=1.25). NF: noise floor, *** p<0.001.

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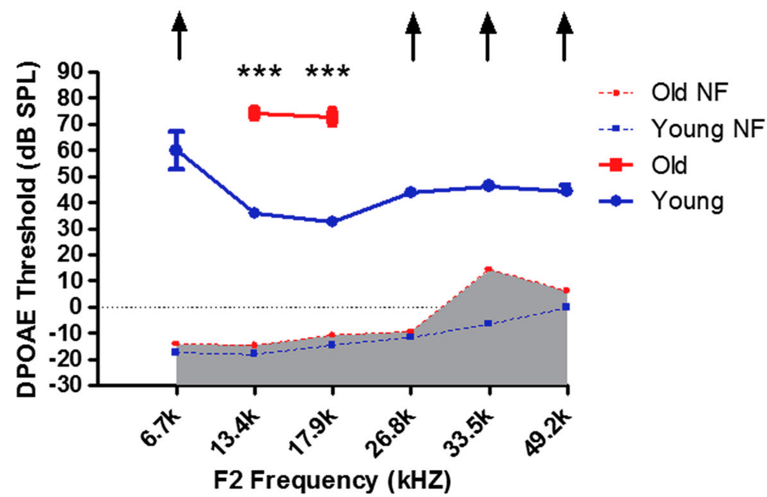

FIG. 3.

DPOAE thresholds of young adult mice are much lower than for old mice. DPOAE (F1=65 dB SPL, F2=50 dB SPL f2/f1=1.25). NF: noise floor,*** p<0.001. Arrows indicate the thresholds exceeded the upper limits of the TDT DPOAE system.

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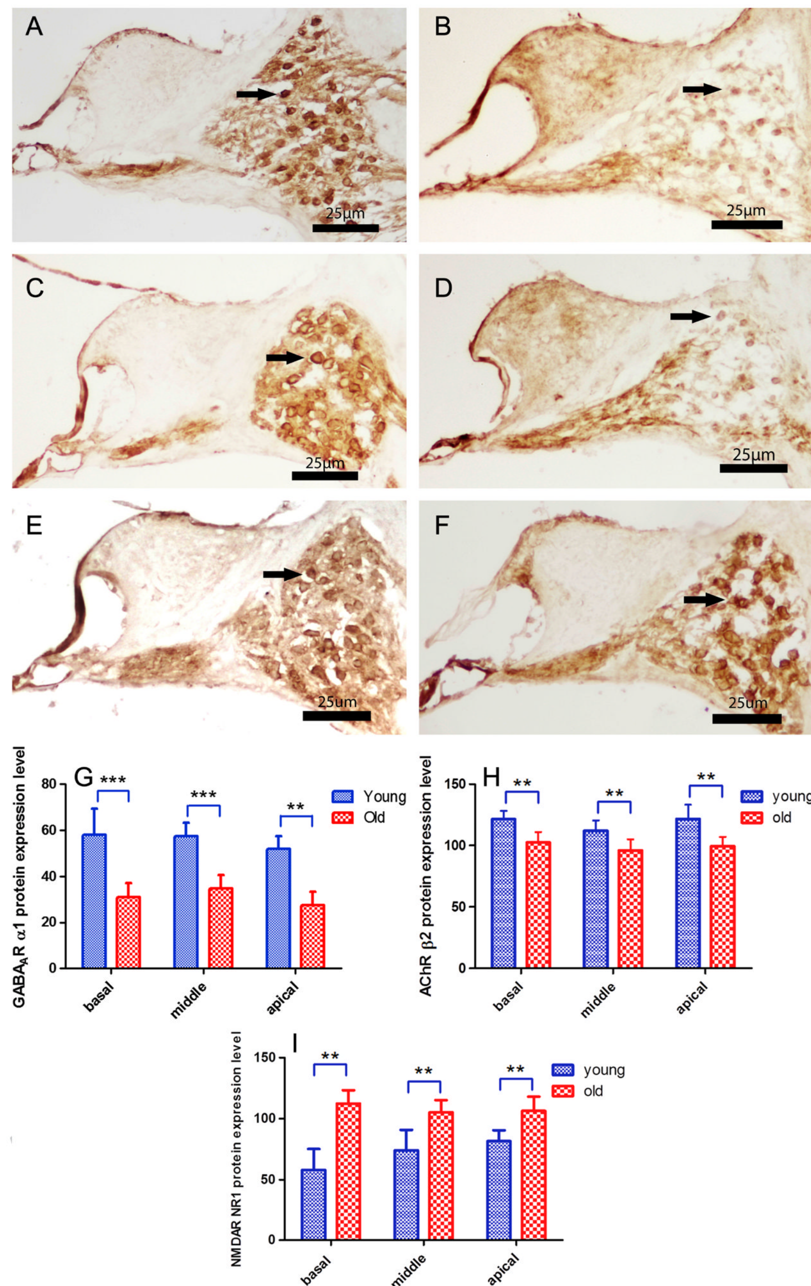

FIG. 4.

Age-related changes of protein expression levels in SGNs. DAB staining (A, B, C, D, E,F) of serial sections from the cochlear middle turn. (A, B) GABA$_A$Rα1 protein expression decreased with age. (C, D) nAChRβ2 protein expression declined with age. (E, F) NMDARNR1 protein expression *increased* with age. (G, H, I,) GABA$_A$Rα1, nAChRβ2 and NMDARNR1 protein expression in basal, middle and apical turns in young adult and old age mice. Bar indicate means ± s.d. for young adult (2–3 month) and old mice (24–32 month). *p<0.5, **p<0.01. Arrow: labeled SGNs. Scale *Bar:* 25 μm.

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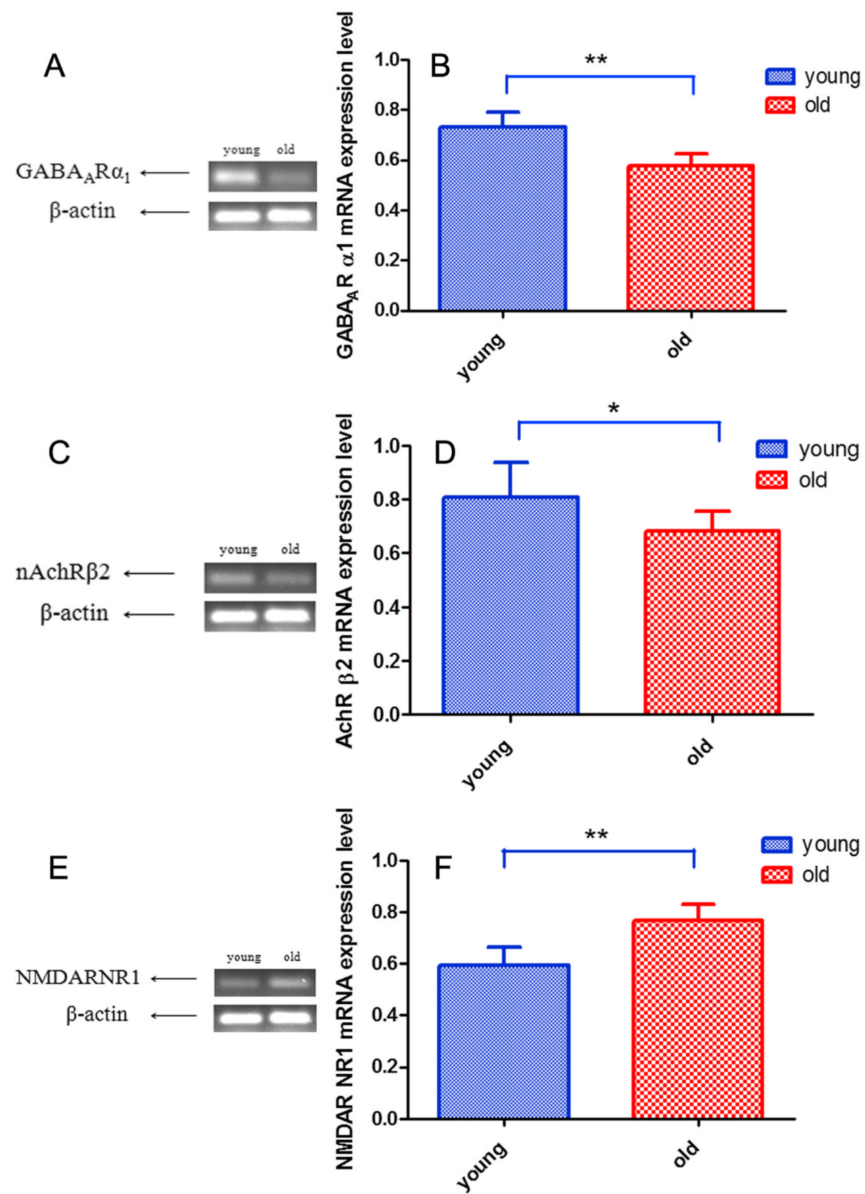

FIG. 5.

Age-related changes of mRNA expression in SGNs. (A, B) GABA$_A$R α1 mRNA expression decreased with age. (C, D) nAChR β2 mRNA expression declined with age. (E, F) NMDAR NR1 mRNA expression *increased* with age. Bar indicate means ± s.d. Young adult (2–3 month), old mice (24–32 month). $^{**}p<0.01$, $^{***}p<0.001$.

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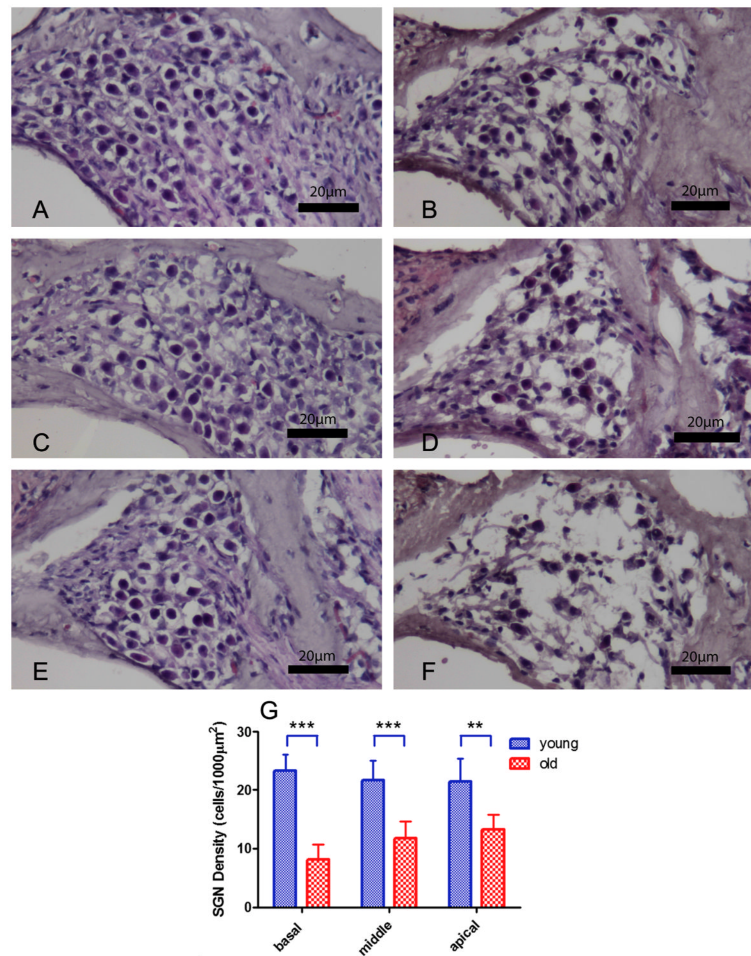

FIG. 6.

H&E staining (A, B [basal turn], C, D [middle turn], E, F [apical turn]) demonstrated spiral ganglion neuron (SGN) loss with age. SGN density declined significantly with age in Rosenthal's Canal (G). Overall SGN density of the basal turn decreased the most. Error bars indicate means ± s.d. Young adult (2–3 month) and old mice (24–32 month). **p*<0.05, ***p*<0.01,*** *p*<0.001. *Scale Bar:* 25 μm.

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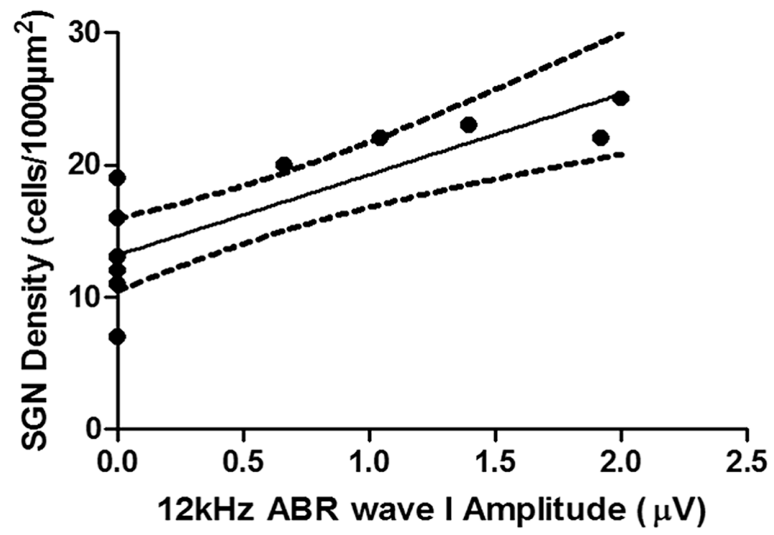

FIG. 7.

Positive correlation between SGN density and 12 kHz ABR wave I amplitude (n=12): r^2=0.7410 , $F_{1,10}$=28.60, p=0.0003.

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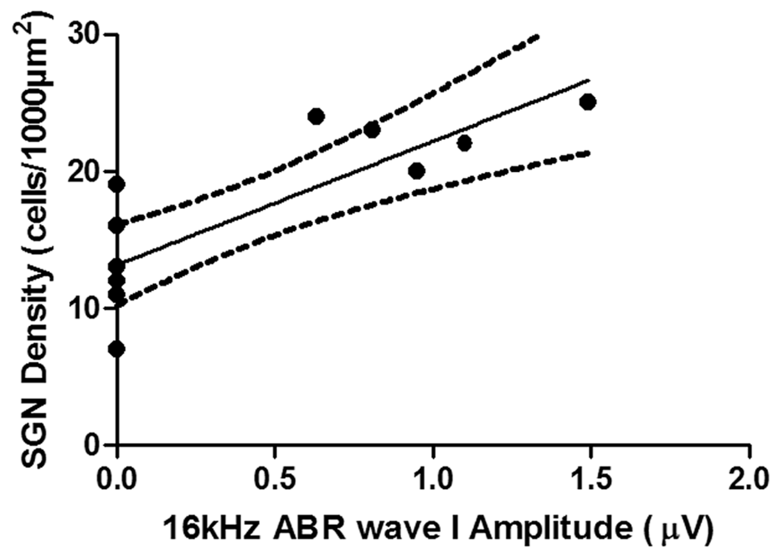

FIG. 8.
Positive correlation between SGN density and 16 kHz ABR wave I amplitude (n=12): r^2=0.6742 , $F_{1,10}$=20.70, p=0.0011.