*Neuroscience* 259 (2014) 184–193

# 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 $GABA_A$ 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 (SGN) of the CBA/CaJ mouse cochlea, that occur in age-related hearing loss, utilizing quantitative immunohistochemistry and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) techniques. We found that auditory brainstem response (ABR) thresholds shifted over 40 dB from 3 to 48 kHz in old mice compared to young adults. DPOAE thresholds also shifted over 40 dB from 6 to 49 kHz in old mice, and their amplitudes were significantly decreased or absent in the same frequency range. 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 $GABA_A$R 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 $GABA_A$R, 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.**


**Key words: aging, hearing loss, cochlea, spiral ganglion neurons, gene expression, protein expression.**

## 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 and Gacek, 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.

GABA is an important inhibitory neurotransmitter in the brain and auditory system. The $GABA_A$ receptor ($GABA_A$R) is a key inhibitory receptor; playing a critical role in regulating neuronal excitability and information processing in the nervous system. $GABA_A$R is a pentameric structure formed by its multi-gene family, and there are at least 19 different members of the $GABA_A$R family, including α1-6, β1-3, γ1-3, ρ1-3, δ1, ε1, θ1, and π1. The majority of $GABA_A$Rs consist of 2α, 2β, and 1γ subunit. In the adult brain, the α1β2γ2

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*Abbreviations:* ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emissions; EDTA, ethylenediaminetetraacetic acid; H&E, hematoxylin and eosin; IC, inferior colliculus; nACh, nicotinic acetylcholine; NMDAR, *N*-methyl-D-aspartate receptor; OHC, outer hair cells; PBS, phosphate-buffered saline; SGN, spiral ganglion neuron; TDT, Tucker-Davis technologies.

0306-4522/13 $36.00 
http://dx.doi.org/10.1016/j.neuroscience.2013.11.058

subtype is the major subtype accounting for 43% of all $GABA_A$Rs (McKernan and Whiting, 1996). The majority of $GABA_A$Rs are widely expressed in the auditory system (Campos et al., 2001; Yamamoto 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 $GABA_A$R 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 $GABA_A$R in the inner ear have not been investigated and it may be that age-related hearing loss is partially due to $GABA_A$R 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 (A–D). 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 and Murrow, 1992). Nicotinic ACh receptors (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 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 and Ashmore, 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 the 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 months) and old age (24–32 months) CBA/CaJ mice to increase our structure–function understanding of pharmacological and electrophysiological characteristics of these receptors.

## EXPERIMENTAL PROCEDURES

### Animals

CBA/CaJ mice were bred in-house under 12:12-h 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. 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).

### ABRs recordings

Mice were anesthetized with a mixture of ketamine/xylazine (120 and 10 mg/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-ms duration and 0.5-ms 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.

### DPOAEs

DPOAEs were obtained following ABR audiometry, under anesthesia (ketamine/xylazine, 120 and 10 mg/kg,