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GABAergic and glutamatergic cells in the inferior colliculus dynamically express the $GABA_AR$ γ_1 subunit during aging

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ABSTRACT

Age-related hearing loss may result, in part, from declining levels of γ-amino butyric acid (GABA) in the aging inferior colliculus (IC). An upregulation of the $GABA_AR$ γ_1 subunit, which has been shown to increase sensitivity to GABA, occurs in the aging IC. We sought to determine whether the upregulation of the $GABA_AR$ γ_1 subunit was specific to GABAergic or glutamatergic IC cells. We used immunohistochemistry for glutamic acid decarboxylase and the $GABA_AR$ γ_1 subunit at 4 age groups in the IC of Fisher Brown Norway rats. The percentage of somas that expressed the γ_1 subunit and the number of subunits on each soma were quantified. Our results show that GABAergic and glutamatergic IC cells increasingly expressed the γ_1 subunit from young age until expression peaked during middle age. At old age (~77% of life span), the number of $GABA_AR$ γ_1 subunits per cell sharply decreased for both cell types. These results, along with previous studies, suggest inhibitory and excitatory IC circuits may express the $GABA_AR$ γ_1 subunit in response to the age-related decline of available GABA.

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1. Introduction

Age-related hearing loss (presbycusis) is one of the most common disabilities in industrialized countries. In the United States, age-related hearing loss doubles every decade of life and affects more than half of the population over the age of 70 years (Bainbridge and Wallhagen, 2014; Lin et al., 2011). Age-related hearing loss can lead to profound deficits in central temporal processing, which in turn can lead to poor speech recognition which can lead to social isolation and depression in an individual (Dalton et al., 2003). γ-Amino butyric acid (GABA), an inhibitory neurotransmitter in the central auditory system, plays a critical role in shaping temporal and spectral responses to fast complex sounds such as speech (Faingold et al., 1989; Frisina, 2001; Le Beau et al., 1996; Pollak et al., 2011). Thus, poor GABA neurotransmission could contribute to various forms of age-related hearing loss.

The inferior colliculus (IC) is a large midbrain nucleus that processes most ascending and descending auditory inputs and integrates temporal and spectral properties for functions such as coding speech and localizing sound. It is generally agreed that the mammalian IC comprises 2 neuronal phenotypes, GABAergic and glutamatergic (Ito et al., 2011; Ito and Oliver, 2012). As demonstrated across multiple species, roughly one-quarter of the cells in the IC are GABAergic (Mellott et al., 2014a; Merchán et al., 2005; Oliver et al., 1994). Numerous investigations have demonstrated that the synthesis of GABA is downregulated with age in the IC (Banay-Schwartz et al., 1989; Burianova et al., 2009; Caspary et al., 1990, 1995, 1999, 2008; Gutiérrez et al., 1994; Helfert et al., 1999; Milbrandt et al., 1994, 1996, 1997; Ouda and Syka., 2012; Rabang et al., 2012; Raza et al., 1994). The downregulation of GABAergic release in the IC is often viewed as a homeostatic response to increase the neural gain due to the diminished excitatory input from the cochlea (Auerbach et al., 2014; Caspary et al., 2008; Gold and Bajo, 2014; Parthasarathy et al., 2018). However, this loss of presynaptic release of GABA may be problematic as GABA in the IC functions to keep precise temporal coding, control gain, and improve signal-to-noise and, in general shape, the acoustic signal (Cai et al., 2014; Frisina, 2001; Palombi and Caspary, 1996; Parthasarathy and Bartlett, 2011; Pollak et al., 2011; Walton et al., 1998, 2002). In addition, the imbalance of inhibition and excitation resultant from enhanced neural gain can interfere with temporal precision and auditory discrimination tasks (Cai and Caspary, 2015; Guo et al., 2017; Overton and Recanzone, 2016; Trujillo and Razak, 2013).

GABAergic inhibition of IC cells is largely mediated by the $GABA_A$ receptor subtype (Kuwada et al., 1980; Palombi and Caspary, 1996; Smith, 1992). $GABA_A$ receptor subunits are heterogeneously expressed across the central nervous system (Burt and Kamatchi, 1991; Pirker et al., 2000; Wisden and Seeburg., 1992). In the rat

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brain, the most common $GABA_A$ receptor subunit combinations are $\alpha_1ß_2\gamma_2$, $\alpha_2ß_{2/3}\gamma_2$, and $\alpha_3ß_n\gamma_{2/3}$ (Caspary et al., 1999; McKernan and Whiting, 1996). However, in situ hybridization studies have shown that the subunit composition of the $GABA_A$ receptor can be drastically altered in the aging IC. Specifically, the $GABA_AR$ γ_1 subunit is conspicuously scarce at young ages but is significantly upregulated with age (Caspary et al., 1999, 2008; Milbrandt et al., 1997). The upregulation of the γ_1 subunit in the IC may have dramatic effects on the physiology of aged IC cells as this subunit has been shown to increase GABA efficacy by increasing the receptor's permeability to chloride (Caspary et al., 1999; Ducić et al., 1995; Dixon et al., 2017; Wafford et al., 1993). Thus, the upregulation of the γ_1 subunit in the IC could be a compensatory mechanism for the demonstrated loss of presynaptic GABA during aging.

In the present study, we used antibodies to an extracellular domain of the $GABA_AR$ γ_1 subunit to assess γ_1 expression and glutamic acid decarboxylase (GAD) to distinguish GABAergic from non-GABAergic (i.e., glutamatergic) neurons in the Fischer Brown Norway (FBN) rat. Our objective was to determine if the age-related upregulation of the $GABA_AR$ γ_1 subunit is specific to either inhibitory or excitatory IC cell populations. The FBN strain is considered a superior model for aging by the National Institute on Aging due to its longer median life span relative to other rat strains and mice and has been used in numerous studies investigating the age-related changes of inhibitory neurotransmission in the peripheral and central auditory systems (Cai et al., 2018; Caspary et al., 2008; Hughes et al., 2010; Ling et al., 2005; Milbrandt and Caspary, 1995; Richardson et al., 2013, 2011; Turner et al., 2005; Wang et al., 2009). Age-related auditory research with rodents often uses two or three "main" age groups, one of which is a young group that can span from 2-8 months of age. Based on preliminary studies, we divided "young" animals into 2 age groups, "young adult-early" (2–3 months) and young adult-late (4–7 months), which revealed a significant increase in expression of the $GABA_AR$ γ_1 subunit during the traditionally identified "young" group. We show that both GABAergic and glutamatergic cells in the IC upregulate the $GABA_AR$ γ_1 subunit with age. In the course of these studies, we observed that inhibitory and excitatory IC cells express the $GABA_AR$ γ_1 subunit across multiple phases as (1) expression was significantly higher across all age groups than the young adult-early group, and (2) the number of $GABA_AR$ γ_1 subunits per cell decreased significantly from middle age (19–22 months) to old age (28 months).

2. Materials and methods

2.1. Animals

All procedures were conducted in accordance with the Northeast Ohio Medical University Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Results are described from 16 FBN rats (National Institute of Aging, Bethesda, MD, USA) of both sexes weighing 242–506 g across 4 age groups: "young adult-early" (2–3 months); "young adult-late" (4–7 months); "middle age" (19–22 months); "old age" (28 months). Efforts were made to minimize the number of animals and their suffering.

2.2. Perfusion and tissue processing

Each animal was deeply anesthetized with isoflurane and perfused transcardially with 0.9% saline solution, followed by 250 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and then by 250 mL of the same fixative with 10% sucrose. The brain was removed and stored at 4 °C in fixative with 25%–30% of sucrose for cryoprotection. The following day, the brain was prepared for processing by removing the cerebellum and cortex and blocking the remaining piece with transverse cuts posterior to the cochlear nucleus and anterior to the thalamus. The tissue was frozen and cut on a sliding microtome into 40-µm-thick transverse sections that were collected in 6 series.

Putative GABAergic cells and $GABA_AR$ γ_1 subunits were stained with immunochemistry for GAD and $GABA_AR$ γ_1, respectively. The GAD antibody used here has been validated in previous studies (Ito et al., 2007). The $GABA_AR$ γ_1 subunit antibody has been validated to target the extracellular amino acid sequence 121-133 of the subunit (www.biossusa.com). We used NeuroTrace (NT; a fluorescent Nissl stain) to identify neuronal profiles that were GAD-immunonegative and to aid in the determination of subdivisions. The sections were pretreated with 0.1% Triton X-100 for permeabilization and 3% bovine serum albumin and 1.5% normal goat serum to limit nonspecific labeling. Sections were incubated overnight for 1–2 days at 4 °C with rabbit anti-$GABA_AR$ γ_1 polyclonal antibody (Bioss, #bs12078 R, RRID:AB_2753348 diluted 1:100) and mouse anti-GAD monoclonal antibody (GAD67; #MAB5406 Millipore, RRID:AB_2278725, diluted 1:400). After incubation, sections were washed in phosphate-buffered saline and then incubated for one hour at room temperature in a mixture of Alexa Fluor 647–tagged goat antimouse secondary antibody and Alexa Fluor 750–tagged goat antirabbit secondary antibody (to reveal GAD67 and $GABA_AR$ γ_1, respectively; each diluted to 1:100; Life Technologies). Near-infrared markers (AF647 and AF750) were chosen to minimize autofluorescent signals from lipofuscin (a pigment that accumulates in the cytoplasm during aging) and are prominent in standard red and green fluorescent channels (Fig. 1). Finally, sections were washed in phosphate-buffered saline and incubated for 20 minutes in a green (500/525) NT (#N-21480 Molecular Probes diluted to 1:100) to label cell bodies in the IC. Sections were mounted on gelatin-coated slides, allowed to dry, and coverslipped with DPX (Sigma).

2.3. Data analysis

Sections were chosen through the midrostrocaudal region of the IC in which the 3 major IC subdivisions (central IC [ICc], lateral cortex of the IC [IClc], and dorsal cortex of the IC [ICd]) are present. Subdivisions of the IC were delineated by their pattern of NT (Nissl) staining and according to a rat anatomical atlas of the brain (Paxinos and Watson, 1998). Immunostaining revealed GAD-immunoreactive (GAD^+) cells and $GABA_AR$ γ_1-immunoreactive subunits in each of the IC subdivisions. Immunopositive cells and receptor subunits were labeled intensely, making it straightforward to distinguish GAD^+ and GAD-immunonegative (GAD^-) cells that expressed the $GABA_AR$ γ_1 subunit.

Sixteen animals (4 from each age group) with robust immunostaining were chosen for quantification. A transverse midrostrocaudal IC section, containing the left and right IC, was quantified per experiment. Thus, a total of 8 ICs were quantified per age group, totaling 32 quantified ICs in the present study. Every GAD^+ cell in each IC subdivision was quantified. Owing to the immense number of GAD^- cells in the IC, we quantified every GAD^- cell across 2 IC sections (one young adult-early and one old age) to determine if γ_1 subunit expression had a bias to cell size or location. We found that $GABA_AR$ γ_1 subunit expression was not correlated or biased to either cell size or location. In the remaining 30 IC sections (90 IC subdivisions), we quantified a random selection of GAD^- cells and checked that the samples were representative. Each cell was manually plotted with a 63× oil-immersion objective (numerical aperture 1.4) aligned to a Neurolucida reconstruction system (MBF Bioscience, Williston, VT, USA) attached to a Zeiss Axio Imager M2. Each combination of GAD^+ and GAD^- cells and their level (none, sparse, or dense) of $GABA_AR$ γ_1 subunit expression were plotted

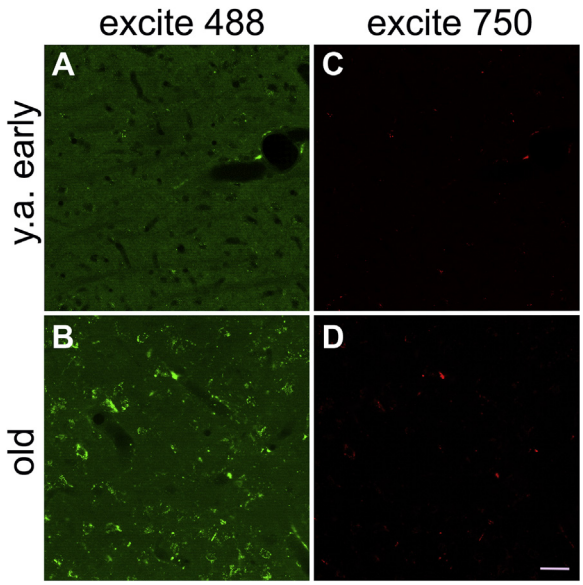

Fig. 1. Autofluorescence that typically accompanies aging is prominent in the green channel ("excite 488") but is nearly invisible in the infrared channel ("excite 750"). In the green (excite 488) channel, lipofuscin fluorescence is low in tissue from the ICc at 3 months of age (A) but very prominent at 28 months (B). (C–D) Tissue autofluorescence, from lipofuscin and other sources, is diminished when the sections shown in A and B are visualized with near-infrared light ("excite 750"). A & C from case R26 and B & D from case R31. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with a unique marker. We counted the fluorescent puncta along or within the perimeter of the plotted cell body and defined the $GABA_AR\ \gamma_1$ expression as "sparse" for 1-10 puncta or "dense" for 11 or more puncta. All photomicrographs presented here are 2-μm maximum image projections and were captured using a Zeiss Axio Imager M2 fluorescence microscope and Hamamatsu ORCA Flash 4.0 camera (Hamamatsu) and optically sectioned at 0.1-μm steps using an Apotome.2 (Zeiss). Adobe Photoshop (Adobe Systems) was used to add scale bars, crop images, erase background around tissue sections, adjust intensity levels, and colorize monochrome images. The results of these plots were used for a quantitative summary of the distribution of GAD^+ and GAD^- cells expressing or not expressing the $GABA_AR\ \gamma_1$ subunit. Final images of the plots were refined using Adobe Illustrator (Adobe Systems, Inc, San Jose, CA, USA) for preparation of figures. Statistical analysis was performed by importing the data into R (version 3.1.2 for Mac OS X; R Core Team, 2014) for chi-squared analysis for independence.

The combined GAD and $GABA_AR\ \gamma_1$ immunostaining often failed to penetrate the full thickness of the section, leaving a central slice of the section unstained. By analyzing the data with a 63X objective (NA = 1.4), it was possible to restrict the quantitative analyses to parts of the section that had successful immunostaining (Mellott et al., 2014b). Sections cut at 40–50 μm typically shrink during dehydration such that the final thickness on slides is 20–30 μm. The GAD and $GABA_AR\ \gamma_1$ staining in these sections usually extended 5–10 μm and 4–5 μm from each surface, respectively. Thus, a central layer up to 22-μm thick could be left unpenetrated by both antibodies. By paying special attention to focusing on the center of the soma when plotting the symbol for a particular cell, data points were obtained with sufficient resolution in the z plane (section depth) to allow subsequent filtering of the data by depth. This yielded 2 zones of data from each section (one associated with each surface), in which tissue was well stained with both antibodies, and a central zone that was not well stained. After the data were plotted, the X, Y, and Z coordinates of all markers were exported from Neurolucida to Microsoft Excel and sorted based on the Z coordinate. Markers in the poorly stained central layer were excluded from further analyses. Such depth filtering necessarily reduces the sample size for quantitative comparisons, but a sufficient number of sections were analyzed to yield a substantial number of cells; 20,836 cells across the 4 age groups were analyzed and coded for $GABA_AR\ \gamma_1$ expression.

3. Results

We combined immunolabeling for GAD and $GABA_AR\ \gamma_1$ to identify phenotype(s) that express the $GABA_AR\ \gamma_1$ subunit with age in the 3 major subdivisions of the IC. We first describe the percentage of GAD^+ and GAD^- cells that express the $GABA_AR\ \gamma_1$ subunit and then describe the results of sparse and dense expression of the $GABA_AR\ \gamma_1$ subunit during aging. Results are described from 10,409 GAD^+ cells and 10,427 GAD^- cells across 16 cases, 4 per age group (Table 1).

3.1. GAD^+ and GAD^- cells expressing the $GABA_AR\ \gamma_1$ subunit

GAD^+ and GAD^- cells and $GABA_AR\ \gamma_1$ subunits were readily identified in each IC subdivision of each age group. As described in Methods, the quantitative analysis was restricted to tissue depths that were successfully penetrated by both antibodies. Therefore, we interpret GAD^- cells as presumptively glutamatergic and not the result of poor GAD staining.

3.1.1. Central nucleus of the IC

In each age group, a subpopulation of GAD^+ and GAD^- cells in the ICc expressed the $GABA_AR\ \gamma_1$ subunit (Fig. 2, arrows). The expression of the $GABA_AR\ \gamma_1$ subunit was conspicuously low at 3 months and was increased at later ages (compare Fig. 2A, D, G, J). The percentage of GAD^+ cells in the ICc that expressed the $GABA_AR\ \gamma_1$ subunit at young adult-early, young adult-late, middle age, and old age was 16%, 49%, 73%, and 64%, respectively (Fig. 3A). Similarly, the percentage of GAD^- ICc cells that expressed the $GABA_AR\ \gamma_1$ subunit at young adult-early, young adult-late, middle age, and old age was 18%, 46%, 76%, and 63%, respectively (Fig. 3B). $GABA_AR\ \gamma_1$ subunit expression, regardless of phenotype, was significantly ($p < 0.001$) increased from young adult-early to young adult-late and then again at middle age (Fig. 3). During middle age, the percentage of cells that expressed the $GABA_AR\ \gamma_1$ subunit had more than tripled from 2-3 months, and the increased expression of the $GABA_AR\ \gamma_1$ subunit was conspicuous (compare Fig. 2A–G). However, at old age, the number of GAD^+ and GAD^- ICc cells expressing the $GABA_AR\ \gamma_1$ subunit was significantly ($p < 0.001$) decreased when compared with that at middle age (Fig. 3). Immunofluorescent γ_1 puncta were often in apposition to immunofluorescent GAD puncta (Fig. 2C, F, I, L; insets). Immunofluorescent γ_1 puncta were also often on the soma of the cell (Fig. 2F, I, L; arrows). Numerous γ_1 puncta were not associated with a soma and were observed in the neuropil, presumably present on astrocytes or unlabeled dendrites.

3.1.2. Lateral and dorsal cortex of the IC

$GABA_AR\ \gamma_1$ subunit expression in the IClc and ICd was similar to the expression observed in the ICc in that (1) expression was relatively low at 3 months and was more robust at later ages (Fig. 3) and (2) a subpopulation of GAD^+ and GAD^- cells expressed the $GABA_AR\ \gamma_1$ subunit in each age groups (Figs. 4 and 5 arrows). The percentage of IClc and ICd cells that expressed the $GABA_AR\ \gamma_1$ subunit, regardless of phenotype, was significantly ($p < 0.001$) increased from young adult-early to young adult-late and then again at middle age (Fig. 3). In contrast to what was observed in the ICc,

Table 1
Summary of cases, age groups, and cell counts expressing the $GABA_AR\ \gamma_1$ subunit in the subdivisions of the IC

A. GAD+ cells; n = 10,409						
y.a.-early	ICc (γ_1 +)	ICc (γ_1 −)	IClc (γ_1 +)	IClc (γ_1 −)	ICd (γ_1 +)	ICd (γ_1 −)
R3 (3 mo, M)	59	376	80	290	71	236
R13 (3 mo, F)	28	131	37	94	30	89
R21 (3 mo, F)	41	207	57	160	50	135
R26 (3 mo, M)	53	238	55	181	49	130
y.a.-late						
R5 (4 mo, M)	93	107	137	129	136	132
R9 (5 mo, M)	47	44	88	54	50	37
R19 (7 mo, F)	55	63	88	73	68	64
R24 (4 mo, M)	59	48	103	71	72	79
Middle age						
R4 (22 mo, M)	234	94	188	83	127	83
R22 (19 mo, F)	127	66	114	60	62	61
R27 (21 mo, M)	138	35	144	34	39	31
R32 (21 mo, M)	108	34	32	28	131	36
Old age						
R20 (28 mo, F)	181	100	340	94	219	85
R31 (28 mo, M)	161	82	288	86	211	107
R33 (28 mo, M)	127	92	273	95	154	80
R38 (28 mo, M)	105	46	193	101	242	84
Totals	1616	1763	2217	1633	1711	1469
	ICc total = 3379		IClc total = 3850		ICd total = 3180	
B. GAD- cells; n = 10,427						
y.a.-early	ICc (γ_1 +)	ICc (γ_1 −)	IClc (γ_1 +)	IClc (γ_1 −)	ICd (γ_1 +)	ICd (γ_1 −)
R3 (3 mo, M)	61	374	55	304	48	240
R13 (3 mo, F)	35	120	32	101	27	74
R21 (3 mo, F)	22	249	20	27	16	136
R26 (3 mo, M)	92	193	91	148	68	135
y.a.-late						
R5 (4 mo, M)	105	99	138	126	143	119
R9 (5 mo, M)	31	68	83	130	31	77
R19 (7 mo, F)	49	78	69	82	57	76
R24 (4 mo, M)	59	47	103	87	72	76
Middle age						
R4 (22 mo, M)	255	86	112	99	133	85
R22 (19 mo, F)	158	55	70	64	82	61
R27 (21 mo, M)	150	47	66	50	78	30
R32 (21 mo, M)	134	32	111	86	69	42
Old age						
R20 (28 mo, F)	180	85	340	109	243	87
R31 (28 mo, M)	164	102	288	124	163	102
R33 (28 mo, M)	133	86	234	106	169	98
R38 (28 mo, M)	95	65	200	84	226	116
Totals	1723	1786	2012	1727	1625	1554
	ICc total = 3509		IClc total = 3739		ICd total = 3179	

Key: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; IC, inferior colliculus; ICc, central inferior colliculus; ICd, dorsal cortex of inferior colliculus; IClc, lateral cortex of inferior colliculus.

from middle age to old age, the percentage of $GABA_AR\ \gamma_1$ subunit expressing cells in the IClc and ICd continued to increase (Fig. 3).

3.2. Sparse and dense expression of the $GABA_AR\ \gamma_1$ subunit

The number of immunofluorescent γ_1 puncta per cell was conspicuously variable. As described in the Methods section, we categorized GAD^+ and GAD^- cells that expressed $\geq$11 γ_1 puncta as dense, 1-10 γ_1 puncta as sparse, and 0 γ_1 puncta as none (Fig. 6; red arrows: dense, white arrows: sparse, arrowheads: none). Of the 5529 GAD^+ cells and 5256 GAD^- cells that expressed the $GABA_AR\ \gamma_1$ subunit in the present study, 1458 and 1298 were classified as dense, respectively.

Figures 7 and 8 show the distribution of GAD^+ and GAD^- cells that did or did not express the $GABA_AR\ \gamma_1$ subunit across 4 age groups, respectively. Note that Figures 7 and 8 are the same IC sections from the same cases. Populations of GAD^+ & γ_1^+ cells (red circles) and GAD^+ & γ_1^- cells (black circles) were distributed in each IC subdivision and largely overlapping (Fig. 7). Populations of "dense" (large red circles) GAD^+ & γ_1^+ cells were commonly distributed evenly across the IC at young adult-early, young adult-late, and old age; however, at middle age, most were found in the ICc (Fig. 7). The pattern of distribution for the GAD^- & γ_1^+ cells (red squares) and GAD^- & γ_1^- (black squares) was very similar to the GAD^+ populations (compare Figs. 7 and 8).

For all 3 IC subdivisions, the highest percentage of GAD^+ & γ_1^+ cells that were classified as dense occurred during middle age: ICc, 48%; IClc, 32%; ICd, 29% (Fig. 9A). The highest percentage of GAD^- & γ_1^+ cells that densely expressed the $GABA_AR\ \gamma_1$ subunit in the 3 IC subdivisions also occurred during middle age: ICc, 45%; IClc, 32%; ICd, 22% (Fig. 9B). Perhaps the most revealing data of the present study were that the percentage of dense GAD^+ and GAD^- cells in each subdivision more than doubled from young adult-early to middle age months and then plummeted from middle age to old age (Fig. 8). During middle age, 48% of the GAD^+ and 45% of the GAD^- ICc cells that expressed the $GABA_AR\ \gamma_1$ subunit were classified as dense; during old age, each of those percentages fell to under 20% (Fig. 9). Significant decreases of dense cells also occurred in the IClc and ICd from middle age to old age (Fig. 9). These data suggest that the $GABA_AR\ \gamma_1$ subunit, a potential compensatory mechanism for the age-related loss of GABA, is substantially downregulated

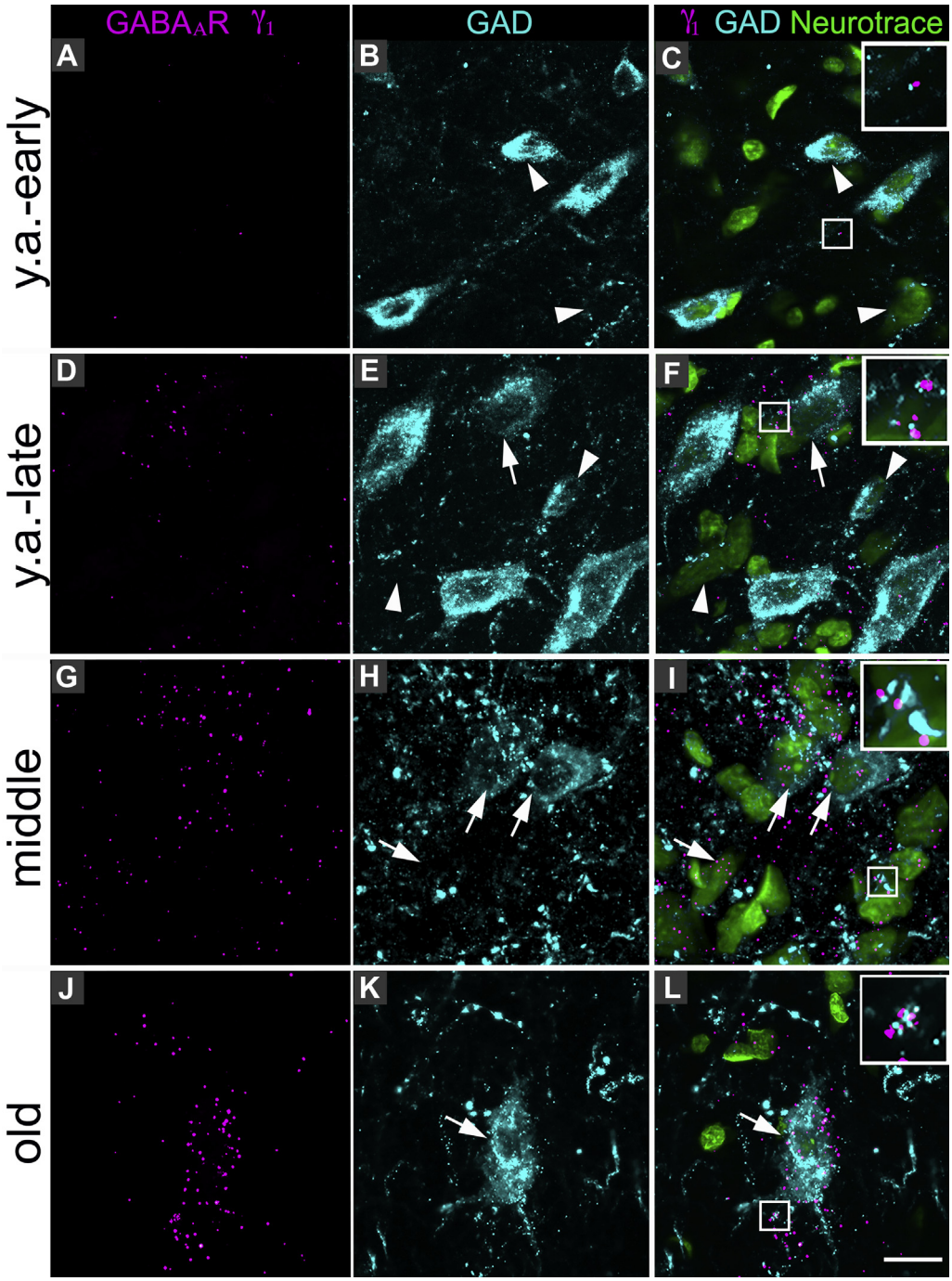

Fig. 2. GAD$^+$ and GAD$^-$ ICc cells express the GABA$_A$R γ_1 subunit throughout aging. Structured illumination fluorescence images, taken at 0.1-μm steps, showing GAD$^+$ and GAD$^-$ ICc cells that express (arrows) or do not express (arrowheads) the GABA$_A$R γ_1 subunit. The GABA$_A$R γ_1 subunit is shown in magenta, GAD is shown in cyan, and NT is shown in green. Each row represents the ICc across a single age group. (A–C) Photomicrographs of a young adult-early ICc. Few GAD$^+$ or GAD$^-$ cells express the GABA$_A$R γ_1 subunit: case R21. (D–F) Photomicrographs of a young adult-late ICc: case R5. (G–I) Photomicrographs of a middle age ICc. Most GAD$^+$ or GAD$^-$ cells in this age group express the γ_1 subunit: case R4. (J–L) Photomicrographs of an old-age ICc: case R34. GABA$_A$R γ_1 subunit puncta can be observed next to GAD puncta in C, F, I and L (Large square insets are magnified views of the smaller square). See list of abbreviations. Scale bar = 20 μm. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; ICc, central inferior colliculus; NT, NeuroTrace. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

across the IC at an age when hearing thresholds are commonly reported to be significantly elevated in rat.

4. Discussion

The present study examines the age-related changes of GABA$_A$R γ_1 subunit expression on GABAergic and glutamatergic cells in the IC. Our first finding is that the percentage of GAD$^+$ and GAD$^-$ cells that expressed the GABA$_A$R γ_1 subunit increased significantly from young adult-early to young adult-late and again at middle age. In the ICc, the percentage of cells that expressed the GABA$_A$R γ_1 subunit from middle age to old age decreased while percentages in the IClc and ICd increased. Interestingly, the expression of the GABA$_A$R γ_1 subunit on GABAergic and glutamatergic cells was remarkably similar across age groups and IC subdivisions (e.g., 64% of old-age GAD$^+$ and 63% of old-age GAD$^-$ cells in the ICc expressed the

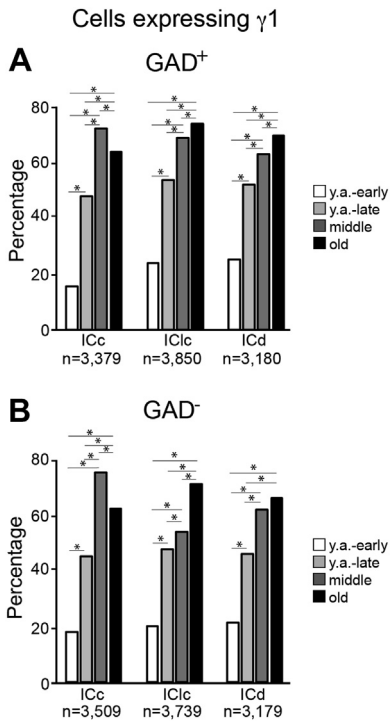

Fig. 3. Histograms summarizing the percentage of cells in the IC that express the γ_1 subunit across the 4 age groups. (A) Percentage of GAD^+ cells that express the γ_1 subunit in the ICc, IClc, and ICd ($^*p < 0.001$). (B) Percentage of GAD^- cells that express the γ_1 subunit in the ICc, IClc, and ICd ($^*p < 0.001$). The number of ICc cells expressing the γ_1 subunit declined between middle and old age. The number of IClc and ICd cells expressing the γ_1 subunit increased between middle and old age. Significance was determined with a chi-squared analysis for independence. Abbreviations: GAD, glutamic acid decarboxylase; IC, inferior colliculus; ICc, central inferior colliculus; ICd, dorsal cortex of inferior colliculus; IClc, lateral cortex of inferior colliculus.

$GABA_AR$ γ_1 subunit; Fig. 3). Our second finding was that when the $GABA_AR$ γ_1 subunits were quantified per soma, it was clear that the expression appeared to be dynamic throughout life. The percentage of $GABA_AR$ γ_1–expressing cells that expressed more than 10 subunits per cell increased from young adult-early to young adult-late until it peaked at middle age. From middle age to old age, the percentage of cells densely expressing the $GABA_AR$ γ_1 subunit was significantly less. However, the expression of the $GABA_AR$ γ_1 subunit in old age was still higher than the young adult-early. The reduction of $GABA_AR$ γ_1 subunits occurred across both phenotypes in each of the major IC subdivisions. In the following sections, we discuss technical aspects of our analysis and then consider some functional implications of $GABA_AR$ γ_1 subunit upregulation.

4.1. Technical considerations

Imaging immunofluorescent signals, in particular receptor subunits that yield small punctate fluorescence, in aging tissue is technically challenging. Lipofuscin, an autofluorescent pigment, appears in the cytoplasm as early as 3–4 months of age and continues to accumulate until death. To better resolve the $GABA_AR$ γ_1 subunit and GAD signals, we used fluorescent tags visible in near-infrared wavelengths where the autofluorescence of the lipofuscin in the IC is diminished (Fig. 1).

Incomplete penetration of immunoreagents can lead to false-negative staining, which could substantially affect quantitative analyses. We systematically limited our analysis such that labeled structures were analyzed only at tissue depths that included robust immunostaining from both antibodies.

We used the fluorescent Nissl stain NT, which labels both neurons and glia, to identify GAD^- somas. To avoid quantifying glia, we (1) excluded cells that were small and had a very small cytoplasm to nucleus ratio and (2) quantified tissue with 3 individuals to cross-validate our neuron numbers. It is possible that a few glia were included in our analysis; however, given the large number of cells quantified in the present study, a few glia cells would not have a substantial effect on our results. GAD and NT immunohistochemistry only label the soma and occasionally a few proximal dendrites. Therefore, the present study was limited to the analysis of somas even though $GABA_AR$ γ_1 subunits are likely expressed on dendrites. It would be interesting to determine if the age-related dendritic upregulation of the $GABA_AR$ γ_1 subunit was specific to either GABAergic and glutamatergic IC cells as it could reflect (1) differences in the patterns of lost GABAergic input and (2) substantial differences in the neurotransmission of GABA between the 2 aging subpopulations.

4.2. Functional implications

4.2.1. $GABA_AR$ γ_1 subunit expression

In younger animals, (i.e., those typically used in research not focused on aging) the $GABA_AR$ γ_1 subunit is largely expressed in the substantia nigra, pallidum, medial and central amygdaloid nuclei, the bed nucleus of the stria terminalis and cerebellar astrocytes; the $GABA_AR$ γ_1 subunit is noticeably minimal or absent throughout most neurons of the central auditory system (Araki et al., 1992; Laurie et al., 1992; Persohn et al., 1992; Pirker et al., 2000; Riquelme et al., 2002; Wisden and Seeburg., 1992). Interestingly, it was discovered that proteins for the $GABA_AR$ γ_1 subunit is upregulated with age in the IC, the largest subcortical nucleus of the auditory system (Caspary et al., 1999; Milbrandt et al., 1997). We also found that the $GABA_AR$ γ_1 subunit is upregulated with age in the IC; however, our results of subunit expression during old age may differ from the previous investigations. Caspary et al., (1999) reported that the protein levels for the $GABA_AR$ γ_1 subunit increase in the FBN rat from young (4 months) to middle age (20 months) to old age (32 months). Our data demonstrate a significant increase of $GABA_AR$ γ_1 subunit expression from young to middle age (19–22 months), but then, they show a decrease in the number of $GABA_AR$ γ_1 subunits per soma from middle age to old age (28 months). It is possible that the signaling pathway for translocating, trafficking, and inserting the $GABA_ARs$ into the plasma membrane of the cell is disrupted in aged cells. This could explain why Caspary et al. (1999) observed an increase in $GABA_AR$ γ_1 subunit protein levels in old age, while our data quantifying $GABA_AR$ γ_1 subunit expression, via an antibody that targets the extracellular domain of the $GABA_AR$ γ_1 subunit, declines with age. Helfert et al., (1999) demonstrated that the age-related loss of GABAergic input to IC cells may predominately be occurring on the tertiary dendrites of the cells. If the expression of the $GABA_AR$ γ_1 subunit is a compensatory mechanism to the age-related loss of GABA, it is possible that the upregulation of the $GABA_AR$ γ_1 subunit occurs more robustly on the dendrites of IC cells rather than on the soma. This could also explain some of the differences observed between the study by Caspary et al. (1999) and the present study (that did not quantify the expression of $GABA_AR$ γ_1 subunits on dendrites). In addition, in the cerebellum, the $GABA_AR$ γ_1 subunit is expressed on astrocytes that wrap inhibitory synapse (Riquelme et al., 2002). It is possible that some of the γ_1 puncta in the present study are being expressed on astrocytes; whether glial expression of the γ_1 subunit changes with age is unknown.

Traditionally, age-related studies of inhibition in the central auditory system routinely define and group rodents between 2–8 months as a singular age group (see reviews Caspary et al., 2008; Syka, 2010). Our finding that the expression of the $GABA_AR$ γ_1 subunit roughly doubles from 2–3 months to 4–7 months of age

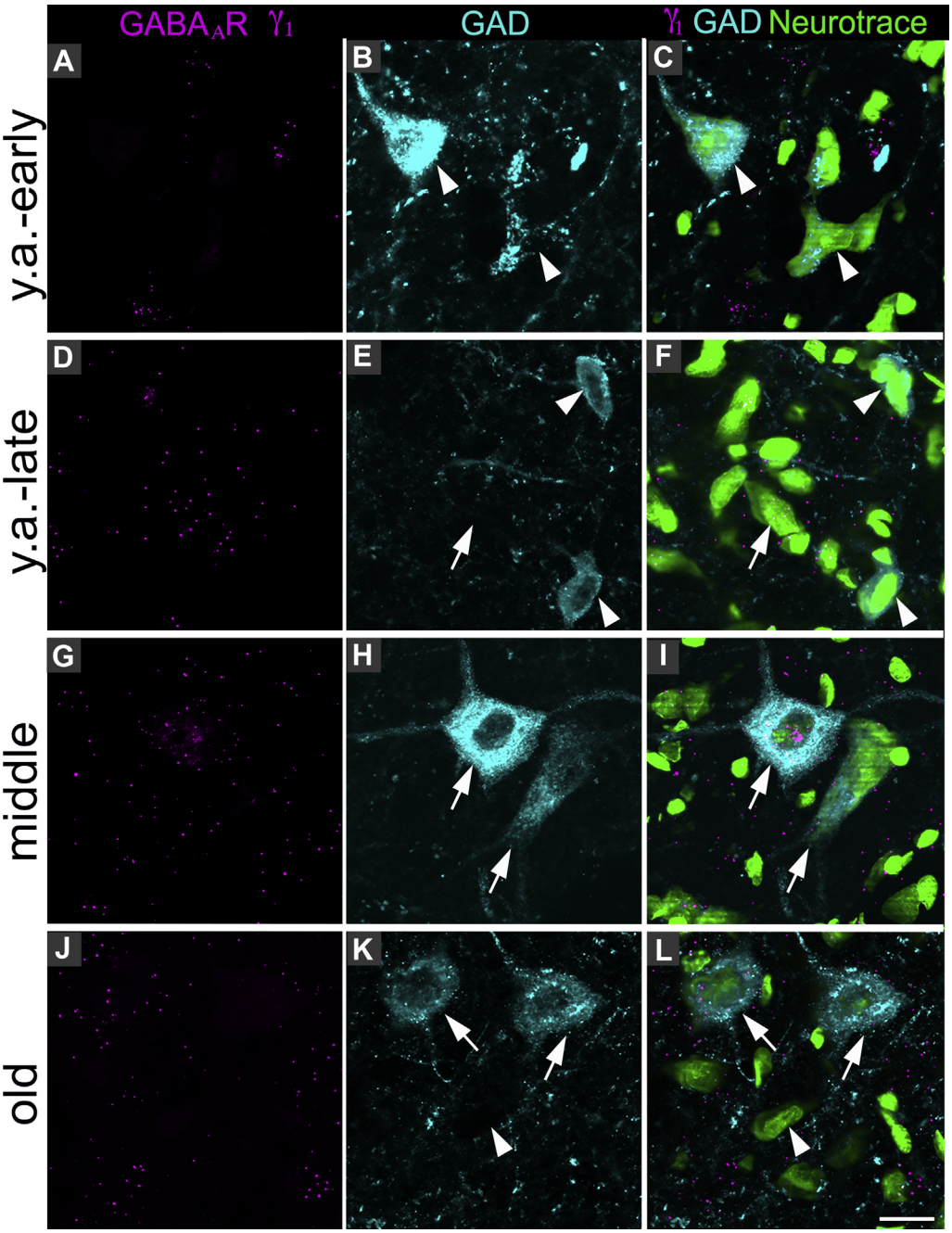

Fig. 4. GAD$^+$ and GAD$^-$ IClc cells express the GABA$_A$R γ_1 subunit throughout aging. Structured illumination fluorescence composite images, taken at 0.1-μm steps, showing GAD$^+$ or GAD$^-$ IClc cells that express (arrows) and do not express (arrowheads) The GABA$_A$R γ_1 subunit is shown in magenta, GAD is shown in cyan, and NT is shown in green. Each row represents the IClc across a single age group. (A–C) Photomicrographs of a young adult-early ICc. Few GAD$^+$ or GAD$^-$ cells express the GABA$_A$R γ_1 subunit. Case R13. (D–F) Photomicrographs of a young adult-late IClc. Case R19. (G–I) Photomicrographs of a middle age IClc. Case R4. (J–L) Photomicrographs of an old age IClc. Case R31. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; ICc, central inferior colliculus; NT, NeuroTrace. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

highlights that aging studies of GABAergic function, auditory or not, may benefit from the use of multiple age groups for "young" animals. Taken altogether, the present study suggests that the IC is likely undergoing substantial changes in GABA regulation and potentially age-related GABAergic loss at a relatively early age.

4.2.2. Aging inhibitory and excitatory IC circuits

It is well known that both GABAergic and glutamatergic IC cells receive GABAergic input (Kelly and Caspary, 2005). We then sought to determine if age-related expression of the GABA$_A$R γ_1 subunit in the IC was specific to inhibitory or excitatory circuits. Given their opposite effects, it would be important to better understand if inhibitory and excitatory IC circuits are compensating for the age-related loss of GABAergic input and if compensation occurs at specific ages and locations in the IC. One could imagine that hearing loss is ultimately reflected in the failure to compensate by either circuit.

We observed the expression of the GABA$_A$R γ_1 subunit on both phenotypes, and to our surprise, they closely paralleled each other. The percentage of cells expressing the GABA$_A$R γ_1 subunit and the

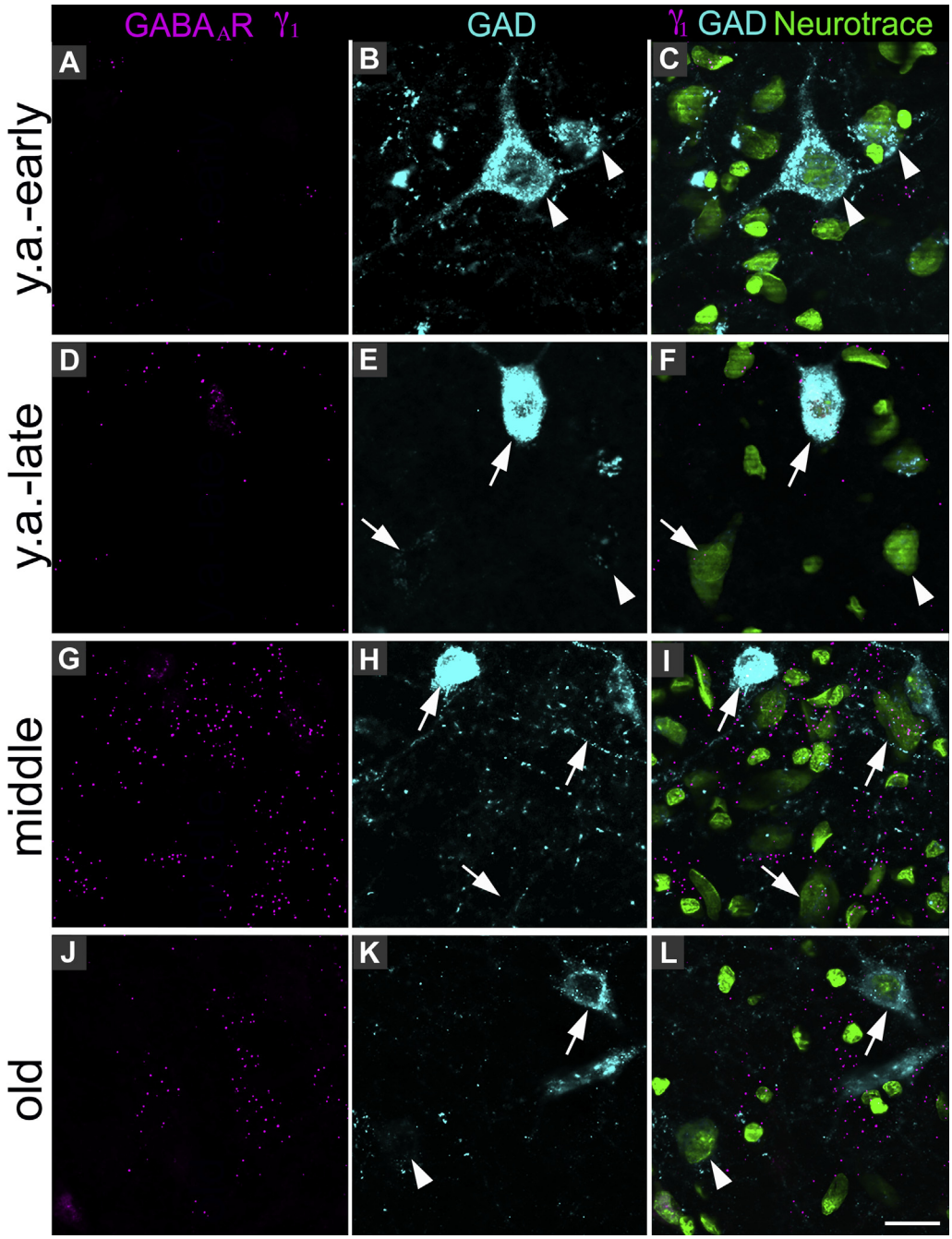

Fig. 5. GAD$^+$ and GAD$^-$ ICd cells express the GABA$_A$R γ_1 subunit throughout aging. Structured illumination fluorescence composite images, taken at 0.1 μm steps, showing GAD$^+$ or GAD$^-$ ICd cells that express (arrows) and do not express (arrowheads). The GABA$_A$R γ_1 subunit is shown in magenta, GAD is shown in cyan, and NT is shown in green. Each row represents the ICd across a single age group. (A–C) Photomicrographs of a young adult-early ICd. Few GAD$^+$ or GAD$^-$ cells express the γ_1 subunit. Case R3. (D–F) Photomicrographs of a young adult-late ICd. Case R9. (G–I) Photomicrographs of a middle age ICd. Case R32. (J–L) Photomicrographs of an old-age ICd. Case R34. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; ICd, dorsal cortex of inferior colliculus; NT, NeuroTrace. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

density in which the subunit was expressed throughout the IC and at various ages were very similar between the 2 phenotypes. Our data imply that inhibitory and excitatory IC circuits are compensating for the age-related loss of GABA largely at the same rate. Inhibitory and excitatory circuits in each major IC subdivisions demonstrated an age-related expression of the GABA$_A$R γ_1 subunit. This would imply that lemniscal and nonlemniscal functions of the IC are likely affected by the age-related loss of GABA. We did observe one primary difference between the lemniscal and nonlemniscal subdivisions. In the ICc, the percentage of cells that expressed the GABA$_A$R γ_1 subunit in old age was significantly less than that at middle age (Fig. 3). In the IClc and ICd, the percentage of cells that expressed the GABA$_A$R γ_1 subunit in old age did not decrease (Fig. 3). Even though there appears to be significant loss of the GABA$_A$R γ_1 subunit in the ICc in old age, it is likely that the overall number of GABA$_A$R γ_1 subunits still result in a compensatory response to a given level of released GABA as Caspary et al. (1999) demonstrated a continuous age-related increase in chloride flux

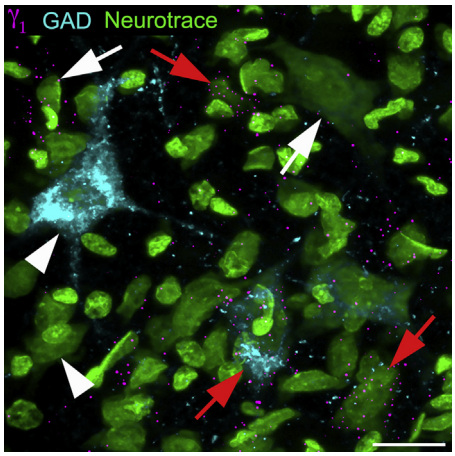

Fig. 6. GAD$^+$ and GAD$^-$ cells have different levels of GABA$_A$R γ_1 subunit expression. Structured illumination fluorescence composite image, taken at 0.1-μm steps, showing GAD$^+$ and GAD$^-$ ICc cells that densely express (red arrows), sparsely express (white arrows), and do not express (arrowheads) the GABA$_A$R γ_1 subunit. The γ_1 subunit is shown in magenta, GAD is shown in cyan, and NT is shown in green. Photomicrograph of an old-age ICc. Case R36. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; ICc, central inferior colliculus; NT, NeuroTrace. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of GABA. The results of the present study and the study by Caspary et al. (1999) give credence to the possibility that the GABA$_A$R γ_1 subunit is increasingly expressed on the dendrites during old age. However, Caspary et al. (1999) examined whole ICs and did not address the subdivisions independently. Therefore, it is possible the increased chloride flux of GABA during old age is a feature of the nonlemniscal IC. Perhaps a decrease of GABA$_A$R γ_1 subunit expressing cells specifically in the ICc plays a role in poorer temporal processing in the lemniscal pathway often reported in those with age-related hearing loss (Frisina, 2001; Palombi and Caspary, 1996; Parthasarathy and Bartlett, 2011; Walton et al., 2002).

4.2.3. Hearing loss

Age-related hearing loss is often characterized as a combination of peripheral and central processing deficits which commonly manifest as temporal processing disorders and difficulties interpreting speech (see reviews of Frisina, 2009, and Liberman, 2017). Whether age-related changes in the central auditory system are a homeostatic process driven by cochlear deafferentation or a de novo and potentially maladaptive response to aging is unclear (Caspary et al., 2008; Caspary and Llano, 2018; Frisina, 2009; Gold and Bajo, 2014). A common view is that the aging central auditory system, in particular the IC, downregulates GABAergic inhibition to compensate for the reduced excitation from the aging cochlea (Auerbach et al., 2014; Caspary et al., 2008; Parthasarathy et al., 2014, 2018; Parthasarathy and Kujawa, 2018). GABAergic input to the IC is essential for dynamic range adjustment, maintaining temporal precision and extracting a signal from noise (Faingold et al., 1989; Frisina, 2001; Palombi and Caspary, 1996; Rees and Møller, 1983, 1987; Vale and Sanes, 2002; Walton et al., 2002). When this GABAergic inhibition is interrupted in the IC, cells lose their precise temporal locking (Frisina, 2001; Le Beau et al., 1996; Palombi and Caspary, 1996; Parthasarathy and Bartlett, 2011; Parthasarathy et al., 2018; Walton et al., 2002). Thus, the cell's ability to follow rapidly time-varying signals, that is, the envelope of speech-like signals, would likely be compromised.

Interestingly, recent studies of noise-induced and age-related hearing loss have demonstrated that the inner hair cells of the cochlea undergo progressive synaptopathy from early in life until old age (Kujawa and Liberman, 2009; Liberman, 2017; Liberman and Kujawa, 2017; Sergeyenko et al., 2013). Therefore, if the lost synapses on the inner hair cells at young ages result in progressively less excitation of the auditory nerve until old age, then the IC may respond by progressively downregulating GABAergic synthesis throughout life in an attempt to maintain a desired level of excitation. Our observation of early increases in γ_1 expression may reflect such ongoing adaptation.

In the present study, we present evidence that inhibitory and excitatory IC circuits dynamically express the GABA$_A$R γ_1 subunit from young adult-early to old age. Given that the GABA$_A$R γ_1 subunit increases GABA efficacy by increasing the receptor's permeability to chloride (Caspary et al., 1999; Ducić et al., 1995; Dixon et al., 2017; Wafford et al., 1993), it has been suggested that this postsynaptic change is a compensatory mechanism in response to the presynaptic age-related loss of GABA in the IC (Caspary et al., 1999; Milbrandt et al., 1997). It appears reasonable that upregulation of the γ_1 subunit is a mechanism that IC cells can use to preserve the levels of inhibition needed for fine temporal processing.

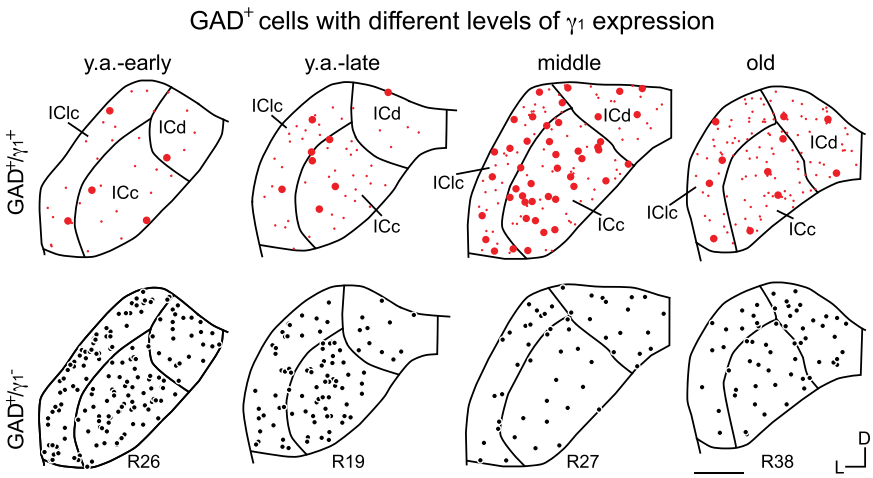

Fig. 7. Distribution of GAD$^+$ cells in the IC at 4 age groups that sparsely expressed the GABA$_A$R γ_1 subunit (small red circles; top row), densely expressed the GABA$_A$R γ_1 subunit (large red circles; top row), or did not express the GABA$_A$R γ_1 subunit (black circles; bottom row). Each symbol represents one labeled cell. D, dorsal; L, lateral. Transverse sections at a midrostrocaudal level of the IC. Scale bar = 1 mm. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; IC, inferior colliculus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

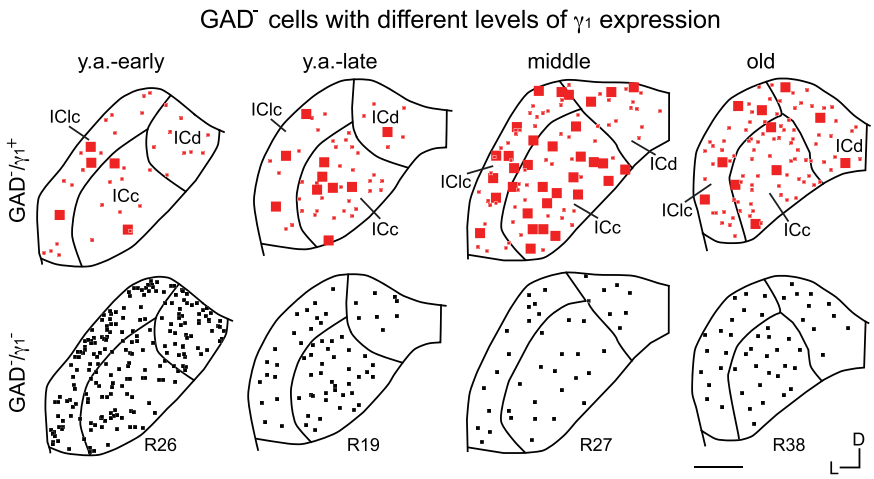

Fig. 8. Distribution of GAD⁻ cells in the IC at 4 age groups that sparsely expressed the $GABA_AR$ γ_1 subunit (small red squares; top row), densely expressed the $GABA_AR$ γ_1 subunit (large red squares; top row), or did not express the $GABA_AR$ γ_1 subunit (black squares; bottom row). Each symbol represents one labeled cell. D, dorsal; L, lateral; Sections are taken in the transverse plane at a midrostrocaudal level. Scale bar = 1 mm. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Our data suggest that the IC may be undergoing age-related changes early in life. Therefore, it is logical to assume that the IC is compensating for the age-related decline of GABAergic input

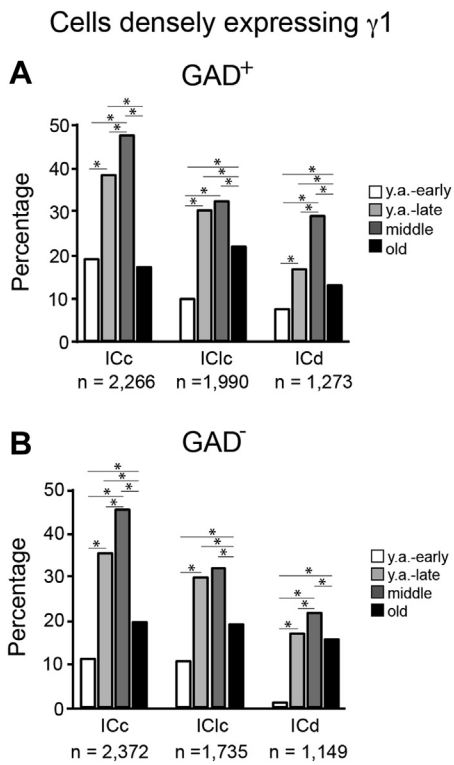

Fig. 9. Histograms summarizing the percentage of $GABA_AR$ γ_1 subunit expressing IC cells across the 4 age groups that densely express the subunit. (A) Percentage of $GABA_AR$ γ_1 subunit expressing GAD^+ cells that densely express the $GABA_AR$ γ_1 subunit in the ICc, IClc, and ICd ($^*p < 0.001$). (B) Percentage of $GABA_AR$ γ_1 subunit expressing GAD^- cells that densely express the $GABA_AR$ γ_1 subunit in the ICc, IClc, and ICd ($^*p < 0.001$). The percentage of dense cells increased significantly from young adult-early to young adult-late and again in middle age. The percentage of dense cells, of both phenotypes in each subdivision, significantly decreased between middle and old age. Significance was determined with a chi-squared analysis for independence. Abbreviations: GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; IC, inferior colliculus; ICc, central inferior colliculus; ICd, dorsal cortex of inferior colliculus; IClc, lateral cortex of inferior colliculus.

rather well for most of life, via the upregulation of the $GABA_AR$ γ_1 or another mechanism(s), as deficits to temporal and speech processing are typically present only in aged and middle-age listeners (Füllgrabe et al., 2014; Pichora-Fuller and Souza, 2003; Ruggles et al., 2011; Walton, 2010). However, the early upregulation of the γ_1 subunit may also reflect a period of synaptic pruning and establishment of higher conductance synapses during young adulthood.

We found a significant reduction in the expression of the $GABA_AR$ γ_1 subunit at 28 months of age which is when hearing thresholds are significantly elevated in the FBN rat (Cai et al., 2018). In particular, we found the greatest loss of $GABA_AR$ γ_1 expression at 28 months in the lemniscal ICc, which is known to receive substantial inhibitory input (Oliver, 2005). It is possible that the initial upregulation in middle age reflects the onset of presynaptic loss, while in old age, there is enough presynaptic loss that there is a coincident loss of postsynaptic γ_1 subunits. Future studies will determine whether the increased expression of the γ_1 subunit during middle age and its subsequent reduction during old age can be correlated to elevated hearing thresholds and deficits.

4.2.4. $GABA_AR$ γ_1 subunit and $GABA_AR$ agonist

Whether the early upregulation and later downregulation of the $GABA_AR$ γ_1 subunit are homeostatic processes or a maladaptive response of the IC cells to aging remains to be determined. Regardless, the presence of the $GABA_AR$ γ_1 subunit is likely to affect the receptor kinetics and overall function of the cell (Caspary et al., 1999; Dixon et al., 2014; Ducić et al., 1995; Ymer et al., 1990). The most common $GABA_AR$ subunit compositions in the IC all contain the γ_2 subunit (Caspary et al., 1999; McKernan and Whiting, 1996). It is the interface between the γ_2 subunit and α_1 subunit of the $GABA_AR$ that allows for the benzodiazepine class of drugs to bind to the receptor and elicit their effect (Burh and Sigel, 1997; Sigel, 2002; Sigel et al., 1998; Wingrove et al., 1997). Thus, $GABA_ARs$ that contain the γ_1 subunit are differentially sensitive as binding of certain benzodiazepines does not enhance synaptic currents (Günther et al., 1995; Wafford et al., 1993; Ymer et al., 1990; Dixon et al., 14). Even though binding levels to agonists of benzodiazepine are minimally affected in the aging IC (Milbrandt et al., 1996), its function is likely affected by the increased presence of the $GABA_AR$ γ_1 subunit. This may in part be due to the fact that levels of mRNA for the $GABA_AR$ γ_2 subunit are not altered with age (Milbrandt et al.,

1997). Thus, a natural question is how GABA sensitivity and levels of inhibition are affected on those cells that express both $GABA_AR\ \gamma$ subunits compared with those cells expressing only the $GABA_AR\ \gamma_2$ subunit. Understanding these relationships and how the physiology of the cell is affected by the presence of the $GABA_AR\ \gamma_1$ subunit is critical as (1) benzodiazepines are routinely prescribed to treat anxiety and depression in individuals with severe age-related hearing loss, noise-induced hearing loss, and tinnitus (Bahmad et al., 2006; Langguth et al., 2018) and (2) pharmacological agents targeting $GABA_AR$ are used to treat hearing deficits (Brecht et al., 2017; Brozoski et al., 2007; Gleich et al., 2003; Sun et al., 2014).

5. Conclusions

The present study demonstrates that the $GABA_AR\ \gamma_1$ subunit is expressed by inhibitory and excitatory IC cells at various ages. Expression of the $GABA_AR\ \gamma_1$ subunit occurred in each major IC subdivision for both phenotypes in each age group. In the lemniscal IC, expression of the $GABA_AR\ \gamma_1$ subunit increased from younger ages until it peaked in middle age. From middle to old age, the number of ICc cells expressing the $GABA_AR\ \gamma_1$ subunit and the number of subunits per ICc cell significantly decreased. In the nonlemniscal IC, the number of cells expressing the $GABA_AR\ \gamma_1$ subunit continued to increase from each age group to the next. However, the number of $GABA_AR\ \gamma_1$ subunits per cell also significantly decreased from middle to old age. Even though there was a reduction in expression in old age, in most cases, the overall expression of the $GABA_AR\ \gamma_1$ subunit was still significantly higher than that in the young adult-early. Interestingly, this reduced $GABA_AR\ \gamma_1$ subunit coincides at an age when hearing thresholds are described as significantly elevated (Cai et al., 2018). Further investigations will be needed to determine if the upregulation of the $GABA_A\ \gamma_1$ subunit is correlated with age-related losses of GABAergic input, and if the later downregulation in old age is correlated with age-related hearing loss or the disturbances of speech interpretation often seen in the elderly.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Disclosure

The authors declare that they have no conflict of interest.

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Author contributions: All authors contributed to data generation and analysis. L.C.R. and J.G.M. designed the experiments. J.G.M. wrote the article.

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