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Laboratory Investigation

Immunoreactivity of Calcium-Binding Proteins in the Central Auditory Nervous System of Aged Rats

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Objective : While many factors contribute to aging, changes in calcium homeostasis and calcium related neuronal processes are likely to be important. High intracellular calcium is toxic to cells and alterations in calcium homeostasis are associated with changes in calcium-binding proteins, which confine free Ca^{2+}. We therefore assayed the expression of the calcium binding proteins calretinin and calbindin in the central auditory nervous system of rats.

Methods : Using antibodies to calretinin and calbindin, we assayed their expression in the cochlear nucleus, superior olivary nucleus, inferior colliculus, medial geniculate body and auditory cortex of young (4 months old) and aged (24 months old) rats.

Results : Calretinin and calbindin staining intensity in neurons of the cochlear nucleus was significantly higher in aged than in young rats ($p<0.05$) The number and staining intensity of calretinin-positive neurons in the inferior colliculus, and of calbindin-positive neurons in the superior olivary nucleus were greater in aged than in young rats ($p<0.05$).

Conclusion : These results suggest that auditory processing is altered during aging, which may be due to increased intracellular Ca^{2+} concentration, consequently leading to increased immunoreactivity toward calcium-binding proteins.

KEY WORDS : Calcium-binding proteins · Aging · Auditory pathway.

INTRODUCTION

Calcium plays a key role as an intracellular mediator of various physiological processes in nerve cells, including their development, growth, transmitter release, transmembrane signaling, and synaptic plasticity[8]. To guarantee the proper functioning of these processes, the concentration of intracellular free calcium must be maintained within an optimal range. Concentrations outside this optimal range often have deleterious, if not fatal, effects on neurons[5,16].

Although many factors contribute to aging, changes in calcium homeostasis and calcium related neuronal processes are likely to be important[14,22]. Age-dependent alterations in the homeostasis of calcium result in changes in the intracellular concentration of calcium ions, which contribute to the neuronal degeneration that often accompanies aging[15]. High intracellular calcium is toxic to cells[19], and alterations in calcium homeostasis are associated with changes in calcium-binding proteins, which confine free Ca^{2+}.

Calretinin and calbindin are cytosolic calcium-binding proteins that are thought to be important regulators of intracellular calcium concentrations[18]. These calcium-binding proteins function to protect neurons from calcium-mediated toxic injury[17,18], due to their capacity to buffer Ca^{2+} and protect against Ca^{2+} overload[10]. Although calcium-binding proteins have been shown to be expressed in the cochlear nucleus and inferior colliculus of many mammalian species[3,7,11,12], less is known about their expression throughout the remainder of the central auditory system. We

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therefore assayed the effect of aging on the expression of calretinin and calbindin throughout the entire central auditory nervous system, including the cochlear nucleus, superior olivary nucleus, inferior colliculus, medial geniculate body and auditory cortex, in young and aged rats.

MATERIALS AND METHODS

Twelve young (4 months old) and ten aged (24 months old) male Wistar rats, bred under specific pathogen-free conditions, were maintained under standard laboratory conditions with a 12 : 12 hour light/dark cycle with free access to food and water. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No.80-23, revised in 1996).

Animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and perfused with 4% freshly prepared paraformaldehyde dissolved in phosphate-buffered saline (PBS) (pH 7.4). The brains were removed and postfixed in the same fixative overnight and subsequently cryoprotected with 20% sucrose in 50 mM PBS, pH 7.4, for 48 hours.

Frozen sections of 40 μm thickness were made in the coronal plane. Non-specific binding sites were blocked by incubation with normal rabbit serum (1 : 50) for 30 minutes at room temperature. The sections were incubated overnight at 4°C with primary antibodies to calretinin and calbindin (Chemicon, Tumecula, CA), washed thoroughly, incubated with secondary antibody for 1 hour at room temperature, and incubated with avidin-biotin complex for 1 hr at room temperature. The bound complexes were visualized by incubating the tissue sections with 0.05% diaminobenzidine and 0.003% hydrogen peroxide.

The atlas of Paxinos and Watson[20] was used for analyses of rat brains. Five slides from the rostral to the caudal level were selected at the same time intervals for each visual region of control and aged rats (cochlear nucleus, Bregma -9.60 to -11.40; superior olivary nucleus, Bregma -9.24 to -10.44;

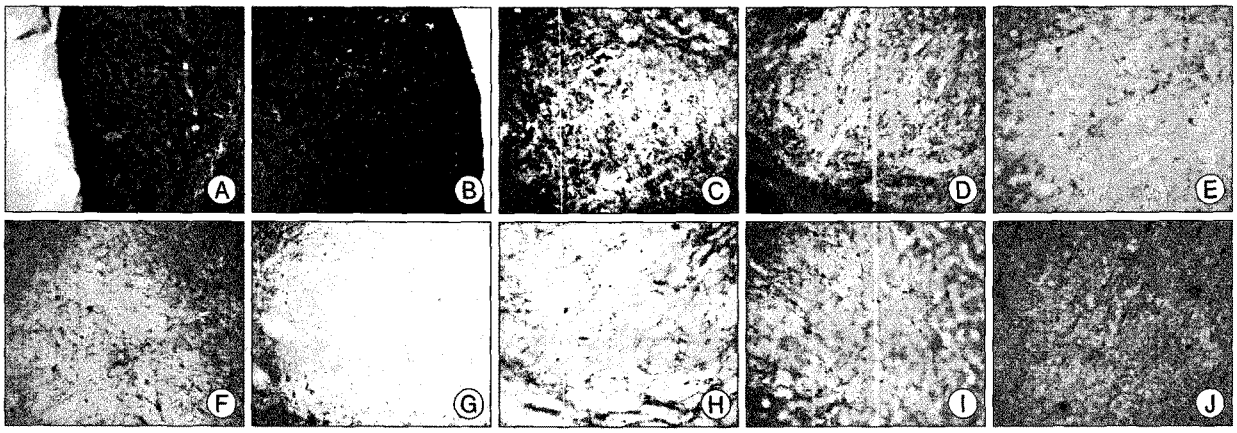

Fig. 1. Calretinin immunoreactivity, Scale bar=100 μm. A : Cochlear nuclei of a young (control) rat. B : Cochlear nuclei of an aged rat. C : Superior olivary nuclei of a young (control) rat. D : Superior olivary nuclei of an aged rat. E : Inferior colliculi of a young (control) rat. F : Inferior colliculi of an aged rat. G : Medial geniculate body of a young (control) rat. H : Medial geniculate body of an aged rat. I : Auditory cortex of a young (control) rat. J : Auditory cortex of an aged rat.

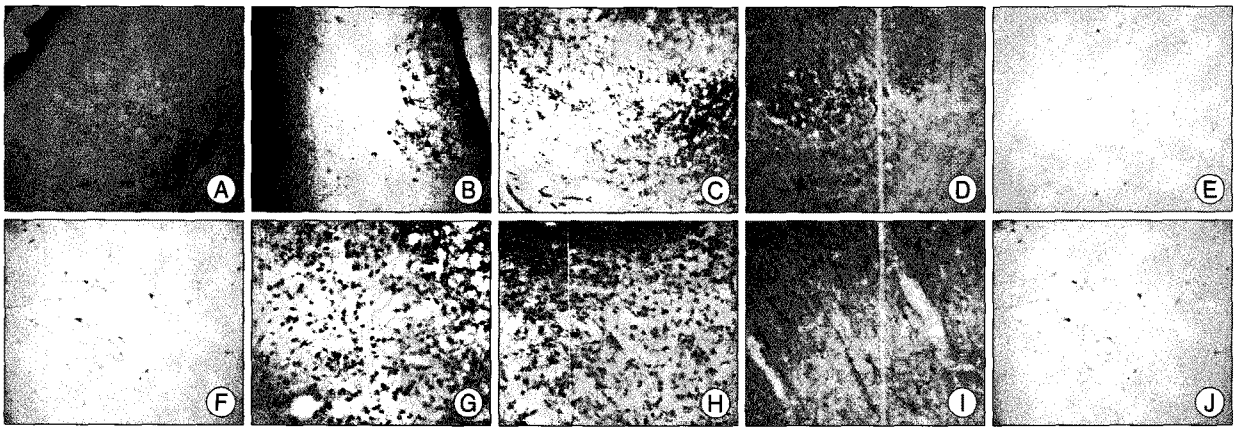

Fig. 2. Calbindin immunoreactivity, Scale bar=100 μm. A : Cochlear nuclei of a young (control) rat. B : Cochlear nuclei of an aged rat. C : Superior olivary nuclei of a young (control) rat. D : Superior olivary nuclei of an aged rat. E : Inferior colliculi of a young (control) rat. F : Inferior colliculi of an aged rat. G : Medial geniculate body of a young (control) rat. H : Medial geniculate body of an aged rat. I : Auditory cortex of a young (control) rat. J : Auditory cortex of an aged rat.

inferior colliculus, Bregma -7.92 to -8.88; medial geniculate body, Bregma -4.92 to -6.24; auditory cortex, Bregma -4.08 to -5.88). Using a Zeiss Axioscope 2 attached StereoInvestigator system (MicroBrightfield, USA), the number of calretinin- and calbindin-positive neurons in each corresponding region of the visual system was counted, with the number of labeled cells expressed per unit area (mm^2). The area and density of stained neurons were measured using an image analysis program (Multiscan, Fullerton, USA), and the average area and signal density at diverse visual regions were calculated in young and aged rats. Student's t-tests were used for statistical comparisons, with $p<0.05$ defined as statistically significant.

RESULTS

Calretinin

Only a few weakly stained cells were observed in the medial geniculate body (Fig. 1). There were significantly more calretinin-positive neurons in the inferior colliculus of aged compared with young rats ($p<0.05$) (Table 1). In all auditory pathway regions examined, however, the area of calretinin-positive neurons did not differ significantly between aged and young rats (Table 2), but the staining density of calretinin-positive neurons was significantly greater in the cochlear nucleus and inferior colliculus of aged compared with young rats ($p<0.05$) (Table 3).

Table 1. Number of calretinin-positive neurons in the rat central auditory nervous system

Location	Control	Aged
Cochlear nucleus	167.3±33.0	170.2±46.8
SON	43.1±9.9	51.2±18.4
Inf colliculus	38.9±16.3	57.2±21.6*
MGB	3.2±3.5	6.7±7.5
Auditory cortex	30.3±9.5	34.1±9.3

Results are presented as the mean±SEM of the number of calretinin-positive neuronal cell bodies per unit area (mm^2). *$p<0.05$. SON : superior olivary nucleus, MGB : medial geniculate body.

Table 2. Area of calretinin-positive neurons in the rat central auditory nervous system

Location	Control	Aged
Cochlear nucleus	49.3±12.3	54.6±17.8
SON	66.9±21.5	58.8±22.8
Inf colliculus	43.9±9.5	44.1±18.1
MGB	51.3±12.7	56.2±14.6
Auditory cortex	37.8±13.2	43.6±19.2

Results are presented as mean±SEM of the area of calretinin-positive neuronal cell body (μm^2). SON : superior olivary nucleus, MGB : medial geniculate body

Table 3. Staining intensity of calretinin-positive neurons in the rat central auditory nervous system

Location	Control	Aged
Cochlear nucleus	118.4±9.9	126.4±7.3*
SON	134.0±6.9	132.5±6.5
Inf colliculus	118.8±8.1	124.6±7.2*
MGB	114.5±15.9	120.8±13.8
Auditory cortex	117.2±11.1	121.3±12.7

Results are presented as mean±SEM of the optical density of calretinin-positive neuronal cell body. The microscopic densitometry results are expressed in arbitrary densitometric units. *$p<0.05$. SON : superior olivary nucleus, MGB : medial geniculate body

Calbindin

In the cochlear nucleus, there were more calbindin-positive than calretinin-positive neurons (Fig. 2). We observed a significantly greater number of calbindin-positive neurons in the superior olivary nucleus of aged than of younger rats ($p<0.05$) (Table 4). In all regions of the auditory nervous system, the area of calbindin-positive neurons was similar in aged and younger rats (Table 5), but the staining density of calbindin-positive neurons in the cochlear nucleus and superior olivary nucleus was greater in aged than in young rats ($p<0.05$) (Table 6).

DISCUSSION

With the alteration in synaptic processing, declines in inhibitory neurotransmitters, and disruption of temporal processing, during aging calcium levels become elevated, resulting in a modification of intracellular signaling pathways, excitability of neurons, neurotransmitter release, gene transcription, and neuronal cell death. As in many other regions of the central nervous system, increased calcium-binding protein immunoreactivity compensates for increased intracellular calcium concentrations in neurons[10,22]. In addition, auditory neurons have among the highest rates of activity in the central nervous system, and their proper function depends on the maintenance of calcium homeostasis[9].

Studies of the expression of calcium-binding proteins in central auditory areas, mainly the cochlear nucleus and the inferior colliculus, have shown that these proteins are up-regulated in the cochlear nucleus during aging[11,12,25]. These central auditory systems interact with peripheral auditory organs, and are influenced by peripheral inputs. The expression of calcium-binding proteins is increased in the cochlear nucleus and is associated with the degeneration of the spiral ganglion, inner hair cells and outer hair cells; moreover, these proteins are up-regulated after cochleotomy[6,13]. Taken together, these results suggest that the central auditory nervous system attempts to compensate for the reduced input from the auditory periphery by a selective

Table 4. Number of calbindin-positive neurons in the rat central auditory nervous system.

Location	Control	Aged
Cochlear nucleus	54.4±49.9	61.8±62.8
SON	16.5±10.7	31.8±16.4*
Inf colliculus	17.6±10.0	20.5±11.2
MGB	495.6±168.1	516.4±166.5
Auditory cortex	61.1±12.9	62.3±20.6

Results are presented as mean±SEM of the number of calbindin-positive neuronal cell body per unit area (mm^2). *p<0.05. SON : superior olivary nucleus, MGB : medial geniculate body

Table 5. Area of calbindin-positive neurons in the rat central auditory nervous system

Location	Control	Aged
Cochlear nucleus	52.3±13.7	55.9±14.6
SON	50.2±27.4	51.1±30.8
Inf colliculus	43.2±11.7	43.7±13.2
MGB	50.5±13.3	52.3±22.9
Auditory cortex	46.8±18.7	48.2±19.8

Results are presented as mean±SEM of the area of calbindin-positive neuronal cell body (μm^2). SON : superior olivary nucleus, MGB : medial geniculate body

Table 6. Staining intensity of calbindin-positive neurons in the rat central auditory nervous system

Location	Control	Aged
Cochlear nucleus	128.9±6.8	137.9±5.2*
SON	124.1±28.9	136.7±3.7*
Inf colliculus	138.3±3.4	136.8±6.3
MGB	130.3±5.7	133.7±4.7
Auditory cortex	114.9±8.6	118.3±12.1

Results are presented as mean±SEM of the optical density of calbindin-positive neuronal cell body. The microscopic densitometry results are expressed in arbitrary densitometric units. *p<0.05. SON : superior olivary nucleus, MGB : medial geniculate body

increase in the expression of calcium-binding proteins.

We assayed the expression of the calcium-binding proteins calretinin and calbindin in aged and young (control) rats by measuring the number, area and density of stained neurons. Although results differed according to protein and assay method, we found that the level of expression of calretinin and calbindin was higher in several central auditory regions of aged rats than control rats. In particular, the staining intensity of neurons in the cochlear nucleus of aged rats was higher for both calretinin and calbindin. In addition, we found that the number and staining intensity of calretinin-positive neurons in the inferior colliculus, and the number and staining intensity of calbindin-positive neurons in the superior olivary nucleus, were higher in aged than in control rats.

Increased expression of calretinin and calbindin in aged rats may be a response to increased intracellular calcium. Age-related alterations in calcium homeostasis may increase intracellular free Ca^{2+} concentration. Since impaired calcium homeostasis may be critical in cellular aging, calcium-binding proteins may have a protective role, due to their buffering capacity in response to increased intracellular Ca^{2+} [22]. Another explanation for the increased expression of calcium binding proteins in aged rats is that these proteins may be markers for aging cells and for rescue of aging cells under age-related stress. For example, it has recently been shown that calcium-binding proteins are newly synthesized in aged rats[6]. A third explanation is that the increased expression of calretinin and calbindin is a response to altered peripheral hearing. Peripheral presbycusis is most likely associated with reduced inhibition in the central auditory circuit, resulting in increased metabolic activity in some central auditory neurons[23,24]. Functional studies have shown that expression of calcium-binding proteins was correlated with metabolic activity and high levels of electrical activity in certain brain regions, and may be responsive to level of neuronal activity[1,2].

In a previous study reporting up-regulation of calretinin expression in the inferior colliculus of old mice, it was suggested that the dorsal cortex of the inferior colliculus may play a critical role in a feedback loop that functions to modify ascending auditory input to the cortex, and that activity within this feedback loop causes calretinin up-regulation in old, but not young, mice[25]. Thus, young mice may have mechanisms of calcium regulation that do not involve calretinin. The inhibitory influences of cortico-fugal projections on the inferior colliculus may decline with age, resulting in increased collicular firing rates and adaptive up-regulation of the calcium buffering system[25].

The medial superior olivary nucleus normally receives direct converging excitatory input from both the ipsilateral and contralateral cochlear nuclei. In contrast, the lateral superior olivary nucleus receives direct excitatory input from the ipsilateral cochlear nucleus, which converges with inhibitory input from the medial nucleus of trapezoid body neurons driven by the contralateral cochlear nucleus[4,21].

Although we found that expression of calbindin was greater in aged than in young rats, there were no side-related differences in expression. Thus, the increased expression of calbindin in aged rats may reflect a modified age-related afferent capacity of both cochlear nuclei, and alterations in excitatory and inhibitory processes.

We found that expression of these calcium binding proteins differed relative to the region of the central auditory nervous system and the type of calcium-binding protein. These region- and protein-specific increases in calcium-binding protein expression suggest that neurons have various strategies for survival against age-related adverse effects of intracellular calcium. Differences in neuronal vulnerability

or buffering capacity between diverse populations of auditory nuclei may be an explanation for this phenomenon.

CONCLUSION

We have shown here that expression of calretinin was higher in the cochlear nucleus and inferior colliculus and that expression of calbindin was higher in the cochlear nucleus and superior olivary nucleus, of aged compared with young rats. These results suggest that auditory processing is altered during aging, and that increased intracellular Ca^{2+} concentrations lead to increases in immunoreactivity of calcium-binding proteins, thus protecting the central auditory system against degenerative changes.

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