Age-related Decrease of GABA_A Receptor Subunits and Glutamic Acid Decarboxylase in the Rat Inferior Colliculus

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A selective age-related decrease in both the protein and mRNA levels of the most abundant GABA_A receptor subunits has been revealed in the rat inferior colliculus. The number (not affinity) of the native and fully assembled GABA_A receptors assayed by ³H-muscimol binding was also decreased (35–49%). The decrease in GABA receptors was accompanied by a decrease in the protein and mRNA of the GABA-synthesizing enzyme glutamic acid decarboxylase. No other region of the rat brain showed such large age-related changes in these GABAergic synaptic molecules. Specific antibodies and riboprobes in conjunction with a computerized image analysis system were used to quantify immunocytochemistry and *in situ* hybridization. In old Sprague–Dawley rats, the combination of β_2 and β_3 peptide subunits was reduced 55%, while the β_2 and β_3 mRNAs were decreased 31% and 22%, respectively. The γ_{2S} and γ_{2L} subunit proteins decreased 43% and 21%, respectively, while the γ_2 mRNA, including both short and long forms, was reduced 61%. The α_1 subunit protein was decreased 28%, whereas the α_1 mRNA decreased 40%. The glutamic acid decarboxylase protein was reduced 62% while GAD₆₅ mRNA decreased 42%. Similar age-related changes were also observed in the inferior colliculus of Fischer-344 rats. In contrast, no changes were observed in the level of expression of some glial and/or neuronal proteins such as S-100, glial fibrillary acidic protein, and 160 KDa neurofilament protein in the inferior colliculus. These results demonstrate the existence of an age-related decline of GABAergic neurotransmission in the rat inferior colliculus that might contribute to some age-related neural auditory dysfunctions.

[Key words: GABA_A receptor, benzodiazepine receptor, glutamic acid decarboxylase, immunocytochemistry, *in situ* hybridization, aging, inferior colliculus, rat]

The inferior colliculus is very important integrative center of auditory information (i.e., frequency, intensity, and binaural differences of acoustic stimuli) in the CNS. The inferior colliculus receives inputs from many ascending and descending auditory pathways. It is rich in inhibitory GABAergic neurons and terminals (Mugnaini and Oertel, 1985; Moore and Moore, 1987; Roberts and Ribak, 1987; Caspary et al., 1990). The major GABAergic sources of the inferior colliculus are the intrinsic GABAergic interneurons (Roberts and Ribak, 1987; Oliver et al., 1991; Oliver and Beckius, 1992) and the extrinsic projection from the dorsal nucleus of the lateral lemniscus (Adams and Mugnaini, 1984; Faingold et al., 1993; Schneiderman et al., 1993). Physiological studies have shown that GABA is an inhibitory neurotransmitter in the inferior colliculus (Faingold et al., 1989, 1991). Moreover, the presence of GABA_A receptors has been determined by radioligand binding (Palacios et al., 1981; Glendenning and Baker, 1988), as well as by immunocytochemistry (Richards et al., 1987; De Blas et al., 1988), immunoprecipitation (Miralles et al., 1994), and *in situ* hybridization (Persohn et al., 1992; Wisden et al., 1992; Miralles et al., 1994). The GABA_A receptors are involved in the normal physiology of the inferior colliculus (Yang et al., 1992; Park and Pollak, 1993). Recently, Caspary et al. (1990) have shown that in the inferior colliculus there is an important age-related reduction in both the number of immunoreactive GABA neurons and the release of the neurotransmitter GABA. This reduction seems to be GABA-specific, since no changes were detected in ACh, glutamate, and aspartate release. It has been proposed that these age-related changes in GABAergic transmission observed in the inferior colliculus might lead to the impairment of auditory perception and sound localization related to neural presbycusis (Willott et al., 1988a,b; Caspary et al., 1990).

The GABA_A receptors are heteropentameric membrane proteins constituting GABA-gated chloride channels. Six isoforms of α subunit (α_1 – α_6), three of β (β_1 – β_3), three of γ (γ_1 – γ_3), one of δ , and two of ρ (ρ_1 , ρ_2) have been identified in mammalian brain (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Lüddens and Wisden, 1991; DeLorey and Olsen, 1992). These are encoded by different genes. In addition, two forms of γ_2 subunit, short (γ_{2S}) and long (γ_{2L}), are generated by alternative RNA splicing (Whiting et al., 1990; Kofuji et al., 1991).

In the present communication we have studied the age-related changes of the GABA_A receptors in the rat inferior colliculus. Immunocytochemistry, *in situ* hybridization, and receptor binding techniques have been used for this purpose. We have focused on the expression of α_1 , β_2 , and γ_2 subunits because they are the most abundantly expressed GABA_A receptor subunits in the inferior colliculus (Persohn et al., 1992; Wisden et al., 1992; Miralles et al., 1994). In addition, we have also studied the age-related changes of glutamic acid decarboxylase (GAD), which is the enzyme that synthesizes GABA, and it is specifically localized in GABAergic cells.

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Materials and Methods

Rats. Two-month-old (young) Sprague–Dawley and Fischer-344, 24-month-old (old) Sprague–Dawley, and 30-month-old (old) Fischer-344 rats (all male) were purchased from Sasco Inc. (Omaha, NE) and the International Center of Aging (Bethesda, MD), respectively.

Membrane preparation and binding assay. The whole (bilateral) inferior colliculi (~25 mg each) from eight young and two old decapitated Sprague–Dawley rats were used for the membrane preparations. Membranes (60 µg) were incubated with 40 nM to 4 µM of ³H-muscimol (20 Ci/mmol; New England Nuclear–Du Pont) for 45 min at 4°C in 50 mM Tris-HCl, pH 7.4, in a total volume of 500 µl. The membrane preparation and the binding assay have been described elsewhere (Mernoff et al., 1983). The nonspecific binding was determined in the presence of 1000-fold concentration of GABA. Scatchard analysis and best fit to a two-binding sites model were calculated with the program LIGAND (Munson and Rodbard, 1980).

Tissue preparation for *in situ* hybridization and immunocytochemistry. Young and old Sprague–Dawley and Fischer-344 rats (two of each age and strain) were decapitated and whole brains were quickly removed, frozen in dry ice, and stored at –70°C until sectioning. Unfixed and frozen brains were sectioned in parasagittal plane (16 µm) in a cryostat. Sections were mounted onto twice gelatin-chromo-alum coated slides and stored at –70°C for either *in situ* hybridization or immunocytochemistry. In addition, for immunocytochemistry, two rats of each age and strain were anesthetized with ketamine-HCl (40 mg/kg), xylazine (3 mg/kg), and acepromazine maleate (1 mg/kg) and perfused through ascending aorta with 4% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate in 0.1 M phosphate buffer (PB), pH 7.4 (PLP; McLean and Nakane, 1974). Brains were immersed in the same fixative for 3 hr at 4°C, followed by 30% sucrose in PB until they sunk, and then frozen in dry ice and stored at –70°C before sectioning. Fixed and frozen brains were sliced (25 µm thick) in the parasagittal plane with a freezing microtome and sections were stored free floating in PB, pH 7.4 containing 0.02% sodium azide at 4°C.

Antibodies. The subunit-specific antibodies to GABA_A receptors were prepared in our laboratory. The monoclonal antibody (mAb) 62-3G1 (De Blas et al., 1988; Vitorica et al., 1988), which recognizes both β_2 and β_3 subunits ($\beta_{2/3}$), has been previously characterized (Park and De Blas, 1991; Park et al., 1991; Ewert et al., 1992). The affinity-purified polyclonal antibodies to α_1 COOH, γ_{2S} and γ_{2L} subunits have been recently reported and characterized (Khan et al., 1994a,b).

The sheep antiserum to rat brain GAD was provided by Dr. Irwin J. Kopin from the National Institute of Health (Bethesda, MD). This antiserum has previously been well characterized (Oertel et al., 1981a,b). It mainly reveals the GAD concentrating in synaptic terminals, and in immunocytochemistry it shows a distribution similar to GAD₆₅ (Kaufman et al., 1991).

Antisera to S-100 (Dako Corp.) and to glial fibrillary acidic protein (GFAP) (generously provided by Drs. Doris Dahl and Amico Bignami from Veterans Administration Medical Center, West Roxbury) and a monoclonal antibody to neurofilament 160 kDa peptide (N160) (Boehringer–Mannheim) were also used.

Immunocytochemical procedure. Cryostat and free-floating sections from young and old Sprague–Dawley and Fischer-344 rat brains (two of each age and strain) were processed for immunocytochemistry as described elsewhere (De Blas, 1984; Sánchez et al., 1991). Antibodies were diluted in PB containing 0.3% Triton X-100 and 0.1% sodium azide. Sections were incubated with (1) one of the primary antibodies: the affinity-purified antibodies (12 µg/ml) anti- α_1 COOH, anti- γ_{2S} , or anti- γ_{2L} , the culture medium of the anti- $\beta_{2/3}$ mAb 62-3G1 diluted 1:100, anti-GAD at 1:20,000, anti-S-100 at 1:1,000, anti-GFAP at 1:1,000, or the mAb anti-N160 at 1:100, for 48 hr at 4°C; (2) the appropriate secondary antibody: sheep anti-rabbit IgG (Sigma), rabbit anti-mouse IgG (Sigma), or rabbit anti-goat IgG (Cappel), all of them diluted 1:100, for 1 hr at room temperature (RT); or (3) the corresponding peroxidase–antiperoxidase (PAP) complex: rabbit serum, mouse monoclonal, or goat serum (all from Sigma), diluted 1:100 for 1 hr at RT. After each incubation, the sections were washed three times with PB for 15 min each. The reaction product was visualized using 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.005% hydrogen peroxide, 0.03% cobalt chloride, and 0.03% nickel ammonium sulfate in PB for 10–15 min. Free-floating sections were mounted onto gelatin-coated slides and air dried. Mounted cryostat or free-floating sections were dehydrated through ethanol gradient, immersed in xylene, and coverslipped with DePeX mounting medium (BDH Laboratory Supplies).

Control sections that were not incubated with the primary antibodies showed no specific immunostaining. The specificity of the immunoreactions obtained with the different GABA_A receptor subunit antibodies has been demonstrated by showing total displacement of the immunostaining by adding the corresponding antigen (synthetic peptide, fusion protein, or affinity-purified GABA_A receptor) to the primary antibody (De Blas et al., 1988; Gutiérrez et al., *in press*).

Riboprobes. The DNAs encoding the intracellular loop located between M3 and M4 putative transmembrane domains of rat β_2 (375 bases) and β_3 (369 bases) subunits (Ymer et al., 1989), bovine α_1 (261 bases) and human γ_2 (260 bases) subunits (Schofield et al., 1987; Pritchett et al., 1989) were used for the preparation of the riboprobes. The procedure for the synthesis of γ_2 and β_2 cRNA riboprobes has been recently reported (Miralles et al., 1994). The α_1 and β_3 riboprobes were generated according to the same methodology. Briefly, the DNAs of the intracellular loop were amplified by PCR using the corresponding subunit cDNA clone as template (Schofield et al., 1987; Pritchett et al., 1989; Ymer et al., 1989) and specific forward and reverse oligonucleotides primers (α_1 , 1208–1229 and 1448–1468; β_2 , 1055–1081 and 1049–1429; β_3 , 1052–1071 and 1399–1419; γ_2 , 1295–1313 and 1537–1555) containing an EcoRI (α_1 , γ_2 , and β_2) or BamHI (β_3) restriction sites at both ends. Amplified DNAs were inserted into either pSP65 plasmid (Promega) for α_1 and γ_2 in both sense and antisense orientations or pBluescript SKII plasmid (Stratagene) for β_2 and β_3 . The orientation of the inserts was determined by PCR by combining primers from the insert and the plasmid. The *E. coli* HB101 and XL-Blue bacterial strains were transformed with the pSP65 and pBluescript plasmids, respectively. The pSP65- γ_2 plasmids were linearized with BamHI and the pSP65- α_1 plasmids with SmaI. The sense and antisense RNA probes for each subunit were obtained with SP6 RNA polymerase. The pBluescript β_2 and β_3 plasmids were linearized with either BamHI or HindIII and XbaI or SmaI, respectively, for generating sense or antisense riboprobes. These were obtained using T3 or T7 RNA polymerase, respectively. The transcription reaction mixture contained 1 µg of plasmid DNA, 10 U of RNA polymerase (Promega), 2.25 µM α -³⁵S-CTP (>1000 Ci/mmol; New England Nuclear–Du Pont), and 125 µM ATP, GTP, and UTP. The full-length GAD₆₅ cDNA was inserted into the EcoRI site of the pBluescript SKII vector (Erlander et al., 1991). Transcription in the sense or antisense orientations was done using T3 or T7 RNA polymerase after plasmid linearization with HindIII or XbaI restriction endonucleases, respectively. The reaction was done with 1 µg of plasmid DNA, 20 U of RNA polymerase, 4.5 µM α -³⁵S-CTP, and 500 µM ATP, GTP, and UTP. Radiolabeled RNAs were purified by phenol-chloroform extraction, ethanol precipitation, and desalting through a P-10 column (Bio-Rad). The specific activity of all riboprobes was approximately 8×10^8 dpm/µg.

In situ hybridization. Cryostat sections from two young and two old Sprague–Dawley and Fischer-344 rat brains were brought to RT for 10 min and fixed for 15 min in freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4, rinsed 2×5 min with PBS, acetylated for 10 min in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0, rinsed with $2 \times$ SSC (standard saline citrate; $1 \times$ SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), dehydrated in increasing ethanol concentration gradient (30%, 50%, 70%, 80%, 95%, 100%), air dried, washed with PBS containing 5 mM MgCl₂ for 5 min, incubated for 8 min with 0.25 M Tris, 0.1 M glycine pH 7.4 and for 15 min with 50% formamide in $2 \times$ SSC at 37°C. Sections were then incubated with the hybridization mixture (200 µl/per slide): 50% formamide, $10 \times$ Denhardt's solution, $10 \times$ dextran sulfate, $2 \times$ SSC, 50 mM dithiothreitol, 500 µg/ml tRNA (Boehringer–Mannheim), and 1.7×10^6 dpm of ³⁵S-labeled cRNA probe. Sections were coverslipped and incubated in a humidified chamber overnight at 37°C. After removal of the coverslips, sections were washed with $1 \times$ SSC, $0.5 \times$ SSC, and $0.1 \times$ SSC containing 25% formamide for 30 min each at 55°C for β_2 and β_3 , and 50°C for γ_2 and α_1 . Sections incubated with GAD₆₅ cRNA probe were washed at 50°C with $2 \times$ SSC (15 min), $1 \times$ SSC (20 min), and $0.5 \times$ SSC (15 min) containing 50% formamide. Sections were dehydrated through ethanol gradient, air dried, and exposed to Hyperfilm β -Max (Amersham).

Control sections were hybridized with the corresponding sense riboprobe. They showed no detectable signal in any brain region (not shown) confirming the specificity of hybridization signal with the antisense probe.

Quantitative analysis. For any given comparison, the hybridized or immunostained sections from young and old rats were processed in parallel with the same batch of radiolabeled RNA probes or antibodies.

Figure 1. GABA_A receptor immunocytochemistry with anti- $\beta_{2/3}$ (mAb 62-3G1) in parasagittal brain sections from young (*A*) and old (*B*) Sprague-Dawley rats. *C* and *D* show the inferior and superior colliculi of young and old rats, respectively, at higher magnification. A large decrease in the immunoreactivity of the inferior colliculus was observed in old rats, while other cerebral areas showed little or no difference between young and old animals. *Cb*, cerebellum; *CPu*, caudate-putamen; *Ctx*, cerebral cortex; *D*, deep cerebellar nuclei; *H*, hippocampus; *Hp*, hypothalamus; *IC*, inferior colliculus; *M*, medulla; *OB*, olfactory bulb; *SC*, superior colliculus; *SN*, substantia nigra; *SuG*, superficial gray layer of the superior colliculus; *Th*, thalamus. Scale bars: *A* and *B*, 2.7 mm; *C* and *D*, 640 μ m.

Sprague-Dawley and Fischer-344 rats (two of each age and strain) were used for quantification. The results for each animal were obtained by averaging the values of two to four sections. Quantifications were performed with a computerized image analysis system. The images of autoradiographs or immunostained sections were taken with a Dage/MTI model 72 CCD camera fitted with an AF Micro-Nikkor 55 mm lens. The camera was connected to a Matrox MVP-AT array processor installed in an 80286-based AT bus PC with an 80287 math coprocessor, a 44 MB Bernoulli box, and a 41 MB hard disk drive running IM3000B image processing and analysis software (Belvoir Consulting, Long Beach, CA). The detector field was (18.15 $\times$ 17.02) mm for the autoradiographs and (15.02 $\times$ 14.07) mm for the immunostained sections. All images were corrected for any shading error due to the fluorescent light box or camera (Inoué, 1985). For the *in situ* hybridization autoradiographs, a standard calibration curve (dpm/mg tissue vs average gray values) was generated with 16 μ m sections of radioactive α -³⁵S-CTP brain paste (Unnerstall et al., 1982). The average gray values of selected anatomical areas of the brain autoradiographs were in the linear portion of the calibration curve. The dpm/mg tissue value in each brain region was calculated from the integrated gray values by the computer program by interpolation. Values were corrected subtracting the corresponding background value taken from the cerebellar white matter. The data were also corrected for the difference in the autoradiography exposure time.

For immunocytochemistry quantification, a standard optical density (O.D.) calibration curve was generated from 11 preset neutral density filters in 0.1 O.D. steps from 0 to 1.0 (Stouffer Graphics Arts Equipment, South Bend, IN). The quantification procedure was similar to the one for *in situ* hybridization.

Results

Immunocytochemistry

Figures 1 and 2 show that in the inferior colliculus of Sprague-Dawley rats there is a significant age-related decrease in GABA_A receptor immunoreactivity. This decrease was observed with all the subunit-specific antibodies used: anti- $\beta_{2/3}$, anti- α_1 COOH, anti- γ_{2S} , and anti- γ_{2L} . Changes in the protein levels (immunoreaction) of these subunits in the whole inferior colliculus (including central, dorsal, and external nuclei) of young and old rats were determined by quantitative image analysis (Table 1). The age-related changes were statistically significant for the $\beta_{2/3}$ and γ_{2S} ($p < 0.01$, Student's *t* test) as well as for α_1 and γ_{2L} subunits ($p < 0.05$). Figure 1 and Table 1 show that there is a 55% reduction of $\beta_{2/3}$ immunostaining in old rats. For the α_1 subunit, the immunoreaction in old rats was decreased 28% (Fig. 2*A,B*; Table 1). The γ_{2L} was reduced 21% (Fig. 2*C,D*; Table 1), whereas γ_{2S} subunit was reduced 43% (Fig. 2*E,F*; Table 1). Anti-GAD immunoreactivity (used as marker for GABAergic synaptic contacts) was reduced 62% (Fig. 3, Table 1). Similar age-related reductions of GABA_A receptor subunits and GAD were also observed in Fischer-344 rats (not shown).

These striking changes were easily noticed in the inferior col-

Figure 2. GABA_A receptor immunocytochemistry with anti- α_1 COOH (A and B), anti- γ_{2L} (C and D), and anti- γ_{2S} (E and F) of the inferior and superior colliculi of young (A, C, and E) and old (B, D, and F) Sprague-Dawley rats. The inferior colliculus of old rats show less immunoreactivity with the three GABA_A receptor subunit antibodies than the inferior colliculus of young animals. IC, inferior colliculus; SC, superior colliculus. Scale bar, 770 μ m.

liculus without the need for quantification. No other brain region showed such dramatic changes. For comparative purposes, we have also quantified the immunoreactivity in a neighboring area, the superficial gray layer of the superior colliculus (SuG). No significant changes in this area were observed with any of the anti-GABA_A receptor subunit or GAD antibodies (Fig. 4, Table 1).

Antibodies to other glial and/or neuronal proteins (S-100, GFAP, and N160) did not reveal any age-related changes for these proteins either in the inferior colliculus or in the SuG (Fig. 4, Table 1), indicating that the observed age-related GABAergic changes in the inferior colliculus are specific and that they do not result from widespread losses of neuronal and/or glial proteins nor they are due to age-related differential tissue penetration of the antibodies.

In situ hybridization

We have designed subunit-specific riboprobes by selecting the large intracellular loop located between the putative transmembrane domains M3 and M4 as the target region for hybridization. The DNA sequences encoding this loop show high subunit specificity (Olsen and Tobin, 1990).

Significant age-related reductions ($p < 0.05$ or $p < 0.01$) in the expression of the mRNAs encoding β_2 , β_3 , α_1 , and γ_2 GABA_A

Table 1. Quantitative immunostaining of Sprague-Dawley rat brain sections

Anti-bodies	Inferior colliculus		Superior colliculus (SuG)	
	O.D.	% Change	O.D.	% Change
Anti- β_2				
Young	0.40 $\pm$ 0.005		0.47 $\pm$ 0.005	
Old	0.18 $\pm$ 0.003	−55**	0.46 $\pm$ 0.003	−2.1
Anti- α_1 COOH				
Young	0.29 $\pm$ 0.010		0.44 $\pm$ 0.005	
Old	0.21 $\pm$ 0.005	−28*	0.43 $\pm$ 0.005	−2.3
Anti- γ_{2L}				
Young	0.24 $\pm$ 0.003		0.34 $\pm$ 0.005	
Old	0.19 $\pm$ 0.003	−21*	0.35 $\pm$ 0.005	+2.9
Anti- γ_{2S}				
Young	0.21 $\pm$ 0.010		0.34 $\pm$ 0.005	
Old	0.12 $\pm$ 0.010	−43**	0.33 $\pm$ 0.005	−2.9
Anti-GAD				
Young	0.29 $\pm$ 0.005		0.39 $\pm$ 0.001	
Old	0.11 $\pm$ 0.005	−62**	0.39 $\pm$ 0.003	0
Anti-S-100				
Young	0.24 $\pm$ 0.007		0.26 $\pm$ 0.010	
Old	0.25 $\pm$ 0.005	+4.2	0.25 $\pm$ 0.005	−3.8
Anti-GFAP				
Young	0.19 $\pm$ 0.005		0.30 $\pm$ 0.001	
Old	0.18 $\pm$ 0.005	−5.3	0.29 $\pm$ 0.005	−3.3
Anti-N160				
Young	0.19 $\pm$ 0.005		0.07 $\pm$ 0.005	
Old	0.19 $\pm$ 0.003	0	0.07 $\pm$ 0.005	0

The optical densities (O.D.) of the whole inferior colliculus (including central, dorsal, and external nuclei) of immunostained tissue sections from young (2-month-old) and old (24-month-old) Sprague-Dawley rats were measured with a computerized image analysis system. The superficial gray layer (SuG) of the superior colliculus was also analyzed as a control area. Data are mean $\pm$ SD of the values collected from two rats of each age.

* $p < 0.05$, Student's t test.

** $p < 0.01$, Student's t test.

receptor subunit were also observed in the inferior colliculus of old Sprague-Dawley and Fischer-344 rats (Fig. 5A–H; Tables 2, 3). The GAD₆₅ mRNA was also reduced (Fig. 5I,J; Tables 2, 3). The reduction of GABA_A receptor subunits and GAD₆₅ mRNAs in the inferior colliculus of old rats were very similar in Sprague-Dawley and Fischer-344 rats (30.5% and 32% for β_2 , 22% and 23% for β_3 , 40% and 36% for α_1 , and 61% and 53% for γ_2 , respectively). The expression of GAD₆₅ mRNA was also reduced 42% and 44%, respectively. In contrast, no changes in the various GABA_A receptor subunit or GAD₆₅ mRNAs were detected in the SuG (Tables 2, 3; Fig. 5).

³H-muscimol binding

Figure 6 and Table 4 show the Scatchard analysis of ³H-muscimol binding to the GABA_A receptor of the inferior colliculus of young and old Sprague-Dawley rats. The binding isotherms (Table 4) were determined by curve fitting to a two-site binding model using the LIGAND program. Others have also shown that ³H-muscimol binds to GABA_A receptor to high- and low-affinity binding sites (Olsen et al., 1981). The B_{max} values of both high- and low-affinity ³H-muscimol binding sites were significantly decreased in the inferior colliculus of the old rats ($p < 0.004$).

Figure 3. GAD immunocytochemistry of parasagittal brain sections from young (*A* and *C*) and old (*B* and *D*) Sprague-Dawley rats. *C* and *D* show the inferior and superior colliculi at higher magnification. Notice the large immunostaining decrease in the IC of old rats compared to other brain areas. The sections in *A* and *B* do not show the same deep cerebellar nuclei. Therefore, the intensity of the immunostaining in these nuclei cannot be compared. For abbreviations, see Figure 1. The arrow indicates the presence of a folded piece of medullar tissue. Scale bars: *A* and *B*, 2.7 mm; *C* and *D*, 640 μ m.

The decrease was larger for the low-affinity (49%) than for the high-affinity binding sites (35%). The K_d values of the high-affinity binding site for young and old rats were similar, 3.3 ± 0.1 nM and 3.5 ± 0.2 nM, respectively. The K_d values of the low-affinity binding sites were also similar in young and old rats (89.4 ± 2.6 nM and 81.9 ± 4.8 , respectively). The small difference in K_d values were not statistically significant ($p > 0.05$).

Discussion

In the present communication we have revealed the existence of a significant and selective age-related reduction of the GABA_A receptor subunits and GAD₆₅ in the rat inferior colliculus. These changes have been detected at both protein and mRNA level in the two rat strains being studied: Sprague-Dawley and Fischer-344. To the best of our knowledge, this is the first time that age-related reductions in the GABA_A receptor subunits and GAD has been reported in the rat inferior colliculus. In addition, the decrease in receptor subunit protein is accompanied by a decrease in the number (B_{max}) of the mature and fully assembled high- and low-affinity GABA_A receptors. These GABAergic changes were not extensive to other neuronal and/or glial proteins of the inferior colliculus. In addition, similar GABAergic changes were not detected in a neighboring brain structure such as the SuG of the superior colliculus.

Previous *in situ* hybridization studies have revealed that β_2 , α_1 , and γ_2 are the most abundant GABA_A receptor subunit mRNAs present in the rat inferior colliculus (Persohn et al., 1992; Wisden et al., 1992; Miralles et al., 1994). In addition, the presence of $\beta_{2/3}$, α_1 , and γ_{2S} and γ_{2L} subunit proteins in the inferior colliculus has been revealed with subunit-specific antibodies (Richards et al., 1987; De Blas et al., 1988; Benke et al., 1991; Miralles et al., 1994; Gutiérrez et al., in press). Nevertheless, in the inferior colliculus, β_2 mRNA is considerably more abundant than β_3 mRNA (Tables 2, 3; Wisden et al., 1992). In addition, we have also studied the age-related changes in GAD₆₅ mRNA. Both GAD₆₅ and GAD₆₇ mRNAs are present in the inferior colliculus (Erlander et al., 1991; Benson et al., 1992). However, we have used only a GAD₆₅ mRNA probe because only GAD₆₅ concentrates in the synaptic terminals, which is the GAD form that is preferentially recognized by the anti-GAD antiserum used in this study (Erlander et al., 1991; Kaufman et al., 1991).

The observed age-related GABAergic decreases in GABA_A receptor subunits and GAD₆₅ might result from specific changes in the GABAergic synaptic circuitry of the inferior colliculus. This notion is consistent with (1) our observation that in the inferior colliculus there are no changes in the levels of the other neuronal and/or glial proteins tested (S-100, GFAP, and N160)

Figure 4. Immunocytochemistry with anti-S-100 (*A* and *B*), anti-GFAP (*C* and *D*), and anti-N160 (*E* and *F*) antibodies in the inferior and superior colliculi of young (*A*, *C*, and *E*) and old (*B*, *D*, and *F*) Sprague-Dawley rats. Similar immunostaining patterns and intensities are observed between young and old rats. IC, inferior colliculus; SC, superior colliculus. Scale bar, 770 μ m.

and (2) previous studies that have shown that in the inferior colliculus there is an age-related decrease of GABA but not of other neurotransmitters: Caspary et al. (1990) have shown that in the inferior colliculus of aged Fischer-344 rats there is an age-related decrease in both the basal and K⁺-induced release of GABA (35–42%), as well as a decrease (36%) in the number of GABAergic neurons as revealed by immunocytochemistry with an anti-GABA antiserum. On the other hand, no changes were detected in the basal or K⁺-induced release of other neurotransmitters such as glutamate, aspartate, and ACh. In a second study, Banay-Schwartz et al. (1989) have shown a significant age-related GABA decrease (22%) in the rat inferior colliculus.

The enzyme GAD₆₅ is concentrated in the axon terminals of the GABAergic cells (Kaufman et al., 1991). Therefore, the age-related decrease in GAD protein detected by immunocytochemistry could result from reduction of inhibitory innervation from either intrinsic and/or extrinsic GABAergic neurons. Nevertheless, the age-related decrease in GAD₆₅ mRNA, which is mostly present in the neuronal somas and dendrites, most likely results from changes in the intrinsic GABAergic neurons that are abundant in the inferior colliculus (Roberts and Ribak, 1987; Oliver et al., 1991). Extrinsic GABAergic inputs could also be involved, such as the projections from the dorsal nucleus of the lateral lemniscus (Adams and Mugnaini, 1984; Faingold et al., 1993; Shneiderman et al., 1993). Other possible extrinsic sources are

Figure 5. *in situ* hybridization reveals the levels of expression of the mRNAs encoding β_2 (*A* and *B*), β_3 (*C* and *D*), α_1 (*E* and *F*), and γ_2 (*G* and *H*) GABA_A receptor subunits and GAD₆₅ (*I* and *J*) in the inferior colliculus of young (*A*, *C*, *E*, *G*, and *I*) and old (*B*, *D*, *F*, *H*, and *J*) Sprague-Dawley rats. A decrease in the expression of all of these mRNAs is observed in the inferior colliculus of old rats. Ctx, cerebral cortex; IC, inferior colliculus; SC, superior colliculus. Scale bar, 1.2 mm.

the brainstem projections from the intermediate and ventral nuclei of the lateral lemniscus, the principal nuclei of the superior olivary complex, the cochlear and periolivary nuclei (Coleman and Clerici, 1987; Schofield and Cant, 1992), as well as the projections from the contralateral inferior colliculus (Coleman and Clerici, 1987; Saldaña and Merchán, 1992) and the auditory cortex (Coleman and Clerici, 1987; Herbert et al., 1991). We have not investigated yet whether any of these areas show age-related decrease of GAD mRNA and/or protein.

The GABA_A receptor and GAD changes that we have observed in the inferior colliculus could easily be detected without quantification. No other region of the brain showed such large changes. Smaller age-related changes might be revealed in other brain areas after quantification. These studies are in progress. Nevertheless, we did not detect any age-related changes in the

Figure 6. Scatchard analysis of ^3H -muscimol binding to inferior colliculus membranes of young (O) and old rats (●). Each data point represents the mean value of ^3H -muscimol binding from two experiments (two different animals) each done in duplicate. Linear regressions of a two-binding site model was calculated by the LIGAND program.

GABA_A receptor subunits nor GAD₆₅ mRNA or protein in the SuG after quantification, which suggests that the age-related changes observed in the inferior colliculus might not be widespread through the brain. In some experiments we could also notice some age-related decrease of $\beta_{2/3}$ and GAD immunoreactivities in other parts of the brainstem such as medulla, pons, and some layers (optic nerve, intermediate, and deep gray

and white layers) of the superior colliculus. More experiments are needed to decide if these differences are statistically significant.

We do not know yet whether the age-related decrease of GABA_A receptors in the inferior colliculus reflects changes in pre- and/or postsynaptic receptors or both. If they are autoreceptors located on GABAergic intrinsic neurons, they could simply reflect the observed age-related losses in the number of intrinsic GABAergic neurons (Caspary et al., 1990) following either GABAergic neuronal death or their lost GABAergic phenotype. If they are postsynaptic receptors they might reflect plastic syn-

Table 2. Quantitative *in situ* hybridization of Sprague-Dawley rat brain sections

Antisense riboprobes	Inferior colliculus		Superior colliculus (SuG)	
	dpm/mg tissue	% Change	dpm/mg tissue	% Change
β_2				
Young	1785 ± 62		622 ± 99	
Old	1241 ± 30	-30.5**	625 ± 46	+0.5
β_3				
Young	734 ± 5		458 ± 2	
Old	573 ± 1	-22**	464 ± 42	+1.3
α_1				
Young	2982 ± 60		616 ± 7	
Old	1801 ± 19	-40**	663 ± 42	+7.6
γ_2				
Young	2070 ± 294		389 ± 21	
Old	800 ± 15	-61.3*	362 ± 16	-7
GAD ₆₅				
Young	3373 ± 45		1953 ± 25	
Old	1962 ± 12	-42**	1874 ± 52	-4

The radioactivity (dpm/mg tissue) was calculated from the O.D. values of the autoradiographic images of the whole inferior colliculus (including central, dorsal, and external nuclei) and the superficial gray layer (SuG) of the superior colliculus from hybridized tissue sections of young (2-month-old) and old (24-month-old) Sprague-Dawley rats. Data are the mean ± SD of values collected from two rats of each age.

* $p < 0.05$, Student's *t* test.

** $p < 0.01$, Student's *t* test.

Table 3. Quantitative *in situ* hybridization of Fischer-344 rat brain sections

Antisense riboprobe	Inferior colliculus		Superior colliculus (SuG)	
	dpm/mg tissue	% Change	dpm/mg tissue	% Change
β_2				
Young	2242 ± 91		639 ± 10	
Old	1533 ± 145	-32*	629 ± 16	-1.5
β_3				
Young	570 ± 27		474 ± 11	
Old	439 ± 14	-23*	453 ± 17	-4.4
α_1				
Young	3046 ± 18		618 ± 6	
Old	1958 ± 55	-36**	656 ± 17	+6.1
γ_2				
Young	2650 ± 5		688 ± 7	
Old	1248 ± 3	-53**	716 ± 28	+4.0
GAD ₆₅				
Young	3375 ± 122		2033 ± 50	
Old	1896 ± 74	-44**	1975 ± 11	-2.8

See Table 2 notes for details; the only difference is the use of Fischer-344 rats.

* $p < 0.05$ Student's *t* test.

** $p < 0.01$, Student's *t* test.

Table 4. Age-related changes in ³H-muscimol binding to the GABA_A receptor in the inferior colliculus of Sprague-Dawley rats

	$\beta_{\max}$ (pmol/mg protein)		K_d (nM)	
	High	Low	High	Low
Young	0.72 ± 0.02	5.4 ± 0.1	3.3 ± 0.1	89.4 ± 2.6
Old	0.47 ± 0.02*	2.8 ± 0.1*	3.5 ± 0.2	81.9 ± 4.8
% Decrease	34.8	49.0	NS	NS

Data are mean ± SD values of two experiments each performed in duplicate. Values were calculated by the LIGAND program. NS, age-related change is not significant.

* Significant difference between young and old animals, $p < 0.004$, Student's t test.

aptic changes in which decreased GAD containing presynaptic input have downregulated the gene expression (transcription and/or translation) of the GABA_A receptor subunits in the post-synaptic cells.

Others (Willott, 1988a,b; Caspary et al., 1990) have hypothesized that the age-related impairment of GABA release in the inferior colliculus might cause the abnormal auditory perception and processing that accompanies neural presbycusis. Our results show that the previously observed age-related GABA decrease in the inferior colliculus results from a reduction in GAD mRNA and protein and that it is also accompanied by a decrease in the number of GABA_A receptors. A loss of GABAergic inhibition during aging may result in cellular dysfunctions, contributing to the abnormal processing of hearing information. The inferior colliculus is involved in sound localization in space (Aitkin, 1986), and it is known that aged Sprague-Dawley rats have problem localizing the source of sound, although they show no deficit in peripheral hearing function nor in motor or visual processing (Brown, 1984). It has been shown that GABA mediates acoustically evoked inhibitory potentials in the inferior colliculus (Faingold et al., 1989, 1991). Inhibitions in the inferior colliculus increase tuning for sound frequency, location, and duration (Faingold et al., 1991; Yang et al., 1992; Park and Pollak, 1993; Casseday et al., 1994). Moreover, in aged mice there is an increase of spontaneous activity of inferior collicular neurons (Willott, 1991). These results have been explained in terms of age-related decreased inhibitory GABAergic transmission. Our results show that this age-related decrease in synaptic inhibition in the inferior colliculus results from decreased levels of GAD and GABA_A receptors. We do not know yet why the GABAergic transmission of the rat inferior colliculus is subjected to such extensive age-related changes.

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