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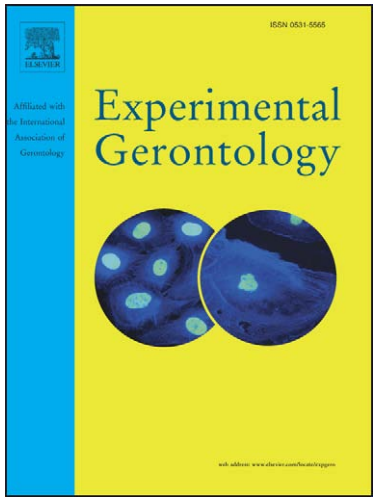

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ABSTRACT

Changes in the levels of gamma-aminobutyric acid (GABA) are known to occur in different parts of the brain during aging. In our study we attempted to define the effect that aging has on glutamate decarboxylase (GAD), the key enzyme in the synthesis of GABA, in the central parts of the auditory system. Age-related changes in GAD65 and GAD67 levels were investigated using immunohistochemistry and western blotting in the inferior colliculus (IC), the auditory cortex (AC) and the visual cortex in Long-Evans rats. The results show that aging is associated with a decrease in the numbers of GAD65- and 67-immunoreactive neurons and the optical density of their somas in both the IC and AC. Western blot analysis revealed a pronounced age-related decline in the levels of GAD65 and 67 proteins in both the IC and AC. For comparison, in the visual cortex the decrease in both proteins was less pronounced than in the IC and AC. A similar pattern of age-related changes was found in Fischer 344 rats, a strain that manifests a rapid loss of hearing function with aging. The observed age-related decline in the levels of GAD65 and 67 may contribute significantly to the deterioration of hearing function that accompanies aging in mammals, including man.

INTRODUCTION

Auditory function in mammals is known to be significantly affected by aging, ultimately resulting in presbycusis (for review see Syka, 2002; Gates and Mills, 2005; Ohlemiller and Frisina, 2008). In the human population, a loss of speech understanding with aging constitutes an important health and social impairment (Frisina and Frisina, 1997; Mazelova et al., 2003; Gordon-Salant et al., 2007). Pathological changes in presbycusis occur both in the inner ear and in the central auditory system. The peripheral component of presbycusis, which comprises mainly alterations of the inner and outer hair cells and/or stria vascularis, is relatively well understood (Schuknecht and Gacek, 1993; Parham et al., 1997; Spongr et al., 1997; Harding et al., 2005; Buckiova et al., 2007). The central component of presbycusis is thought to be associated with age-related alterations in the processing of the temporal parameters of complex acoustical stimuli occurring within the central auditory system. Inhibitory systems in the brain are apparently strongly involved in the temporal processing of acoustical stimuli with the aim of sharpening responses to rapid complex sounds (Walton et al., 1997; Strouse et al., 1998; Krishna and Semple, 2000; Liang et al., 2002; Tremblay et al., 2002; Ostroff et al.,2003; Mazelova et al., 2003). Since gama-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central auditory system (Markram et al., 2004), a decrease in GABA inhibition may significantly contribute to hearing deterioration with aging.

Recently, growing evidence has accumulated about the role of GABA-expressing neurons in inhibitory networks in different parts of the brain (Caspary et al., 1995; Gupta et al., 2000; Buzsáki et al., 2007). The distribution of GABA-immunoreactive (-ir) neurons and inputs in the auditory cortex has been described in detail for cats and monkeys and implies that GABA-ir neurons are present in the AC in relatively large numbers, approximately 20-25% of the total population of neurons (Hendry et al., 1987; Prieto et al., 1994a,b). In the IC, a similar

portion, 20-30% of the total number of neurons, was found to be GABA-ir in cats and rats (Oliver et al., 1994; Merchan et al, 2005). GABA is synthesized by the decarboxylation of glutamate, and the reaction is catalysed by the key rate-limiting enzyme glutamate decarboxylase (GAD). In the mammalian brain, two GAD isoforms of 65 000 and 67 000 molecular weight (GAD 65 and GAD 67) are present (Erlander et al., 1991). Most GABA-expressing neurons contain both isoforms, with GAD 65 being more prevalent in axonal terminals and membranes preferentially synthesizing GABA for vesicular release, while GAD 67 is distributed throughout the neuron including the soma, preferentially synthesizing non-vesicular cytoplasmic GABA (Erlander an Tobin, 1991; Feldblum et al., 1993, 1995; Esclapez et al., 1994; Hendrickson et al., 1994). The two GAD isoforms might be differently involved in the spatial and temporal processing and/or coding of information by GABA-expressing neurons (for review see Soghomonian and Martin, 1998; Wei and Wu, 2008).

Several lines of evidence suggest a decreasing function of GABA-mediated inhibition within the central auditory pathway in aged animals. For example, single unit recordings illustrate the presence of a less precise processing of complex sounds and extended excitatory areas in the IC (Palombi and Caspari 1996; Walton et al., 1998; Walton et al., 2002; Simon et al., 2004). Age-related alterations in the fine-tuned receptive fields and a relative increase in poorly tuned receptive fields could influence the processing of sounds in the central auditory system (Turner et al., 2005a,b). The results of measurements of GABA or GAD levels and GABA receptor subunit levels and binding intensity correspond with the electrophysiological reports. In Fischer 344 rats, a significant age-related decrease in the number of GABA immunoreactive cells was found in the central nucleus of the IC (CIC) combined with a decrease in the enzymatic activity of GAD and in the release of GABA (Caspary et al., 1990; Raza et al, 1994, Caspary et al, 1995). In addition, a decline in the number of GABA-ergic synaptic terminals and synapses of GABA-ir neurons was observed in the CIC with no

detectable neuronal losses (Helfert et al., 1999). Also GABA-A and GABA-B receptor binding intensity was reported to decline, and the protein levels of the receptor subunits were found to be altered during aging in the IC of Sprague-Dawley and Fischer 344 rats (Guttierez et al, 1994; Millbrandt et al., 1994, 1997; Caspary et al., 1999; Schmidt et al., 2008). Decreases in the levels of GAD65 and GAD67 mRNAs and in the optical density of GAD67-immunoreactive cells were observed in the auditory cortex of old Fischer 344/Brown Norway rat (Ling et al., 2005).

However, it is not well known whether the age-related decline in the GABA system is activity-dependent and primarily follows the deterioration of the sensory inputs with aging or whether it rather results from changes occurring within the central auditory system with aging (for review see Caspary et al., 2008). In addition, no clear evidence of inter-strain differences in these changes has been reported, in contrast to the findings in calcium binding proteins that are well known to colocalize in neurons with GABA (Kosaka et al., 1987; Kawaguchi and Kubota, 1998). In this case, a significant strain-specificity in their age-related expression was shown for calretinin and parvalbumin (Zettel et al., 1997; Zettel et al., 2001; Ouda et al., 2008).

In our study we attempted to evaluate the age-related changes in both GAD isoforms in the central auditory system in Long-Evans rats, a strain with a very limited age-related loss of hearing function (Syka et al., 1996; Popelar et al., 2006). Immunohistochemistry and western blot protein analysis were used to compare GAD 65 and 67 expression in the inferior colliculus (IC) and auditory cortex (AC) in young and old rats. In addition, the same analysis was also performed on a representative sample of Fischer 344 rats, a strain with pronounced hearing deterioration with aging, with the aim of studying possible inter-strain differences.

MATERIALS AND METHODS

Animals

Thirty rats, strain Long-Evans (15 young animals 3-5 months old and 15 aged animals 30-35 months old), and twelve rats of the strain Fischer 344 (7 young animals 3-5 months old and 5 aged animals 24 months old) were used in the experiments. The Long-Evans rats were obtained from a local facility and, starting at 2 months of age, were reared and aged (aging group) in-house, under known rearing conditions and with a known health history. Fischer 344 rats were purchased at 2 months of age from Charles River Deutschland (Sulzfeld, Germany) and then reared in-house under the same conditions. No signs of middle ear infection were present in any animal during their stay in the animal facility. The care and use of the animals and all experimental procedures were performed in compliance with the guidelines of the Ethical Committee, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, and the Declaration of Helsinki.

GAD67 immunohistochemistry

Long-Evans rats, 3-5 (n=5) and 30-35 (n=5) months old, and Fischer 344 rats, 3-5 (n=5) and 24 (n=3) months old, were placed under deep anesthesia (ketamine 35 mg/kg + xylazine 6 mg/kg, i.m.) then transcardially perfused with saline followed by 4% paraformaldehyde fixative in 0.1 M phosphate buffer (pH 7.4). Brains were removed within 15 min of perfusion, postfixed 1 hour at 4° C (same fixative) and then cryoprotected with 30% sucrose in phosphate buffer overnight. Coronal sections (40 μm thick) were cut with a freezing microtome.

Free-floating sections were preblocked in normal serum for 1 h and then incubated at 4° C with anti-GAD67 (mouse monoclonal, Chemicon, 1:2000) diluted in PBS containing 1% normal serum. After 24 hours, sections were incubated with a biotinylated secondary antibody (Vector, 1:200) for 45 min and then with peroxidase-labeled ABC reagent (Vector). The

antibody labeling was visualized by incubating the sections for 3 minutes in 0.05% diaminobenzidine (DAB) with 0.01% hydrogen peroxide. Sections were mounted on slides, dehydrated and coverslipped.

GAD65 immunohistochemistry

Long-Evans rats, 3-5 (n=4) and 30-35 (n=4) months old, were subjected to the same procedure leading to the preparation of histological sections as in the case of rats for GAD 67 staining.

Free-floating sections were preblocked in normal serum for 1 h and then incubated at 4° C with anti-GAD65 (rabbit polyclonal, Chemicon, 1:750) diluted in PBS containing 1% normal serum. After 72 hours, sections were incubated with a biotinylated secondary antibody (Biosource, 1:200) for 1 hour and then with peroxidase-labeled ABC reagent (Vector). The antibody labeling was visualized by incubating the sections for 3 minutes in 0.05% diaminobenzidine (DAB) with 0.01% hydrogen peroxide. Sections were mounted on slides, dehydrated and coverslipped.

Western Blot Protein Analysis

Long-Evans rats, 3-5 (n=6) and 30-35 (n=6) months old, and Fischer 344 rats, 4 (n=2) and 24 (n=2) months old, were used in the analysis. Anesthetized rats (ketamine 35 mg/kg + xylazine 6 mg/kg, i.m) were decapitated, their brains quickly extracted and rinsed in ice-cold physiological solution, and the inferior coliculli (ICs) and auditory (ACs) and visual cortices (VCs) were rapidly removed bilaterally. The samples included the whole IC, the Te1+Te3 (Zilles 1995) areas of the AC and the V1+V2 areas of the VC (Paxinos and Watson, 1998). All samples were immediately put into dry ice after extraction and stored frozen at -80°C until processed.

For the analysis, ICs, ACs and VCs were homogenized by a Potter-Elvehjem homogenizer in 0.05 M Tris-NaCl (pH 7.4) buffer with protease inhibitors (Sigma). The homogenate was

centrifuged at 10 000 g for 10 min at 4°C. To ensure similar protein loading, the total protein concentration of these extracts was determined by using the Bradford method with BSA as the standard. Samples (cytosolic fraction) were incubated in boiling water for 10 min at 80°C in sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer containing 10% glycerol, 2% SDS, 0.05% bromphenol blue and 4M dithiotreitol. Samples were then subjected to Tris/Tricine/SDS-PAGE electrophoresis on a 3% bis-acrylamide polyacrylamide gel at 30 mA/gel for 150 min on a Mini-Protean II apparatus (Bio-Rad). After electrophoresis, the resolved proteins were transferred (Bio-Rad Mini Protean II transblot apparatus at 350 mA for 60 min at 4 °C in 25 mM Tris, 192 mM glycine, 20% methanol, 0,1% SDS) to a nitrocellulose membrane (Amersham, Biosciences). Membranes were incubated in 5% non-fat dry milk in 10% Tris-buffered saline with 0.05% Tween 20 (TBST) for 60 min at room temperature to block nonspecific protein binding. After being washed in TBST buffer (3 times quickly, 3 x 5 min each), the membranes were probed with GAD 67-specific (mouse monoclonal, Chemicon, 1:5000 in TBST), GAD 65-specific (rabbit polyclonal, Chemicon, 1:1200 in TBST) or actin-specific (mouse monoclonal, Chemicon, 1:10 000 in TBST) primary antisera overnight at 4°C. The membranes were washed again and incubated with goat anti-mouse IgG or anti-rabbit IgG antibody conjugated with horseradish peroxidase (Upstate, 1:7500; Chemicon, 1:3333 in TBST) for 2 h at room temperature. Before enhanced chemiluminescence (ECL), the membranes were washed as described above and stored in TBST for at least 2 h. For ECL, substrates A (Luminol solution) and B (H_2O_2 solution) were prepared, mixed 40:1 (Amersham Biosciences) just before use, and poured on the membranes. The specific signals were detected on autoradiographic film (Kodak MXB). Films were developed at room temperature, stopped, fixed, washed under running cold water and air-dried. Scanning (Canon CanoScan 8400F) and ImageQuant software were used for quantifying the relative abundance of GAD and actin in individual samples. The amount of

protein applied to the gel varied for each isoform and fraction to achieve linearity with the intensity x area (volume) of the band on the western blot. To ensure the specificity of GAD67, GAD65 and actin immunoreactive proteins, prestained molecular weight protein standards were used (Invitrogen). The levels of GAD65 and GAD67 were calculated as the ratio of the optical density of the antibody of interest to the optical density of the antibody directed against β-actin.

Immunohistochemistry - stereological quantification

GAD65 and 67 immunoreactivity was examined in all sections containing the inferior colliculus, auditory and visual cortices, delineated according to anatomical atlases (Zilles, 1985; Paxinos and Watson, 1998) and with the help of Nissl-stained sections (topographical borders of cortical areas) (Fig. 1A,B). For the IC, sections were sampled from bregma -8.0 mm to -9.2 mm. Sections containing the auditory cortex were sampled from bregma -4.2 mm to bregma -6.0 mm (Zilles, 1995), identified by measuring 2.0-2.5 mm from the rhinal fissure for Te3 and 3.5-4.0 mm for Te1. As an auxiliary criterion, the line going through the dorsal margin of the diencephalon indicated the control dorsal (upper) border of the auditory cortex. For the visual cortex (V1, V2 - Paxinos and Watson, 1998), sections were sampled from bregma -5.0 mm to -8.0 mm. To estimate the total number of GAD65 and 67-ir neurons, an unbiased stereological method, the optical fractionator, was used (West et al., 1991; for review see Mayhew and Gundersen, 1996). The optical fractionator method is unaffected by tissue shrinkage, and therefore it was not necessary to measure this variable. Measurements were determined using bright-field microscopy (Leica DMRXA microscope) connected to a video camera, which transmitted the microscopic image to a monitor (Optronics, 1600x1200 px) coupled with Neurolucida software (MicroBrightField). Two sets of motors were connected to the microscope to move the section a known distance in the x- and y-directions. The z-direction was measured using a microcator. A 10x objective lens was used to delineate

the regions of interest and the starting position of the counting frame. We adapted the Neurolucida-generated grid superimposed on the screen with the use of a random number table to establish the random starting position of the counting frame in the area of interest.

A 100x oil-immersion objective was used and the appropriate counting frame superimposed on the screen. The counting frames used to obtain the sampling had the same size (3880 μm²). The upper and lower surfaces of the sections were examined to determine if the margins of the tissue sections were depleted of neuronal nuclei, as suggested by Andersen and Gundersen (1999). The height of the optical dissector was constant at 5 μm, and the first and last 5 μm of the section thickness was omitted from the analysis. Approximately 200 neurons per animal were counted in each examined structure. Each neuron in the counting frame was counted when its distinct nucleus came to maximum focus. At maximum focus, the cell body was outlined by a cursor on the computer screen, and the morphological parameters and optical density were measured automatically using Neurolucida software. Only positive somas that exceeded 5 μm in diameter and with a distinct nucleus were counted and used for analysis.

Variation in the background immunostaining affects the real values of the optical density of the neuronal soma. In studies of aging, there are not specific “indifferent reference structures” available in the brain for normalization, hence we calculated the normalized value of the optical density in each section as the ratio between the average optical density of the analyzed GAD-ir neurons in the section and the average optical density of a corresponding number of immunonegative cells in the same section (compare Ling et al., 2005). In most cases the immunopositivity of the neurons was explicit; in a few disputable cases, a selected neuron was omitted from the analysis as "non-GAD-ir" unless the measured optical density of its soma was higher than double the average optical density of immunonegative cells in the same section.

Statistical analysis

The results of the statistical analysis were expressed as mean ± S.E.M. The significance of the differences between young and old groups of rats was assessed by the non-parametric two-tailed Mann-Whitney test with the use of GraphPad Prisma software (version 4.0) for both immunohistochemistry and western blots. The significance of differences in western blot protein analysis among protein levels and among the magnitudes of changes in different brain structures (IC vs. AC vs.VC) in young and in old rats was assessed by a one-way ANOVA test and Bonferroni`s multiple comparison test with the use of GraphPad Prisma software (version 4.0). P values of 0.05 or less were considered statistically significant. For each statistical test, each examined animal was represented by one number to ensure the maximal independence of the data entering the tests.

RESULTS

I. GAD65 and 67 changes with aging in the Long-Evans strain

Inferior colliculus

GAD65 and 67-ir neurons were distributed in both young and old animals throughout all three subdivisions of the IC. In the central nucleus of the IC, cell bodies of variable sizes and shapes prevailed, with a large variation in the intensity of immunostaining. The majority of GAD-ir neurons in the external cortex of the IC (EIC) were large spindle and multipolar cells or smaller oval neurons, in both the 2nd and 3rd layers of the EIC (for details see Oliver et al., 1994). Only a few small immunoreactive neurons were present in the dorsal cortex of the IC (Fig.2A,B).

Significant age-related changes in GAD immunoreactivity were found in the CIC and EIC (Fig.3A-D). In the CIC, the optical density of GAD65 and 67-ir neuronal somas decreased by 23% (P=0.028, U=0) and 25% (P=0.008, U=0), respectively. The number of GAD65 and 67-

ir cells decreased slightly by 17% (P=0.028, U=0) and 9% (N.S.), respectively. In the EIC only in GAD67-ir somas the optical density decreased significantly by 14% (P=0.032, U=2), while the number of GAD65 and 67-ir did not differ between old and young animals. No significant age-related changes in the volume of GAD-ir somas were found in the IC with aging.

Western blot analysis demonstrated a significant age-related decline in the levels of GAD65 and GAD67 proteins in the whole IC of old rats in comparison with young animals of 51% and 49%, respectively (both P=0.002, U=0) (Fig. 4). The analyzed samples included all three subdivisions of the IC. A remarkable finding of the additional statistical analysis, in both young and old animals, the levels of GAD65 and GAD67 proteins in the IC were almost three times higher compared to the levels in both the auditory and visual cortices (both proteins P<0.001, Bonferroni`s multiple comparison test).

Auditory cortex

Independently of age, GAD65 and 67-ir cells were scattered throughout all layers, with slightly higher numbers observed in the superficial cortical layers (I – IV) than in the deeper layers (V – VI) (for description see Prietto et al., 1994a,b) The neuropil staining followed the same pattern with greater positivity in the superficial layers (II-IV) (Fig.2C,D).

The results of immunohistochemical staining indicated a significant decrease in the optical density of GAD65 and 67-ir somas in old animals of 21% (P=0.028, U=0) and 22% (P=0.016, U=1), respectively (Fig.3A-D). The number of GAD65-ir neurons did not change in old rats in comparison with young animals (only a slight decrease of 7%, N.S.), while the number of GAD67-ir neurons decreased more markedly by 13% (P=0.032, U=2). No layer-specificity or inter-areal differences (Te1 vs. Te3) of these changes were observed; similarly, the average volume of GAD-ir somas did not change with aging.

Western blot protein analysis revealed a significant age-related decrease in the levels of GAD65 and GAD67 of 43% and 52%, respectively (both P=0.002, U=0) (Fig.4). The analyzed samples included the Te1+Te3 areas. Differences in GAD65 and 67 protein levels between the auditory and visual cortices were non-significant in young animals. In old rats, GAD67 protein levels in the auditory cortex were lower than in the visual cortex (P<0.05, Bonferroni`s multiple comparison test), whereas for GAD65 protein levels, the difference between the auditory and visual cortices was still non-significant. The declines found in all three examined brain regions are apparent on representative blots shown in Fig. 5.

Visual cortex

GAD65 and 67 immunoreactive neurons were present in all cortical layers of the visual cortex; their distribution and neuropil staining followed a similar pattern as in the auditory cortex.

The optical density was found to decrease by 11% for GAD65-ir somas (N.S.) and 18% for GAD67-ir somas (P=0.016, U=1,5) (Fig.3A-D). In contrast, the numbers of GAD65 and 67-ir neurons did not change with aging (the decrease was in the range of 5%, N.S). As in the AC, no significant changes in the average volumes of immunoreactive somas or layer-specificity were found with aging.

In western blot analysis, decreases in the levels of GAD65 and GAD67 proteins were found in the visual cortex as well: a decrease of 22% for GAD65 (P=0.041, U=5) and 20% for GAD67 (P=0.028, U=2) (Fig.4). However, the decrease was less apparent compared to both the auditory cortex and the IC (P<0.01, Bonferroni`s multiple comparison test), while a comparison between decreases in the IC and AC showed no difference. These results held true for both GAD65 and GAD67.

II. GAD65 and 67 changes with aging in the Fischer 344 strain

In principle, the age-related changes in Fischer 344 rats followed the same pattern as the age-related changes in the Long-Evans animals; however, the relatively low number of animals of this strain used in our study precluded a detailed statistical evaluation of the results. Using immunohistochemistry, only differences in GAD67 staining were studied: the optical density of GAD67-ir neuronal somas decreased by 21% (P=0.035, U=0), 13%, 14% and 15% in the CIC, EIC, AC and VC, respectively, while the number of GAD67-ir cells decreased by 6%, 10%, 10% and 6% in the same structures (Fig.3E-F).

Both GAD65 and GAD67 proteins were examined by western blot protein analysis. In the IC, GAD65 protein levels decreased by 46% and GAD67 levels by 44% (Fig. 4). The decline of GAD65 protein levels in the AC was 34% and that of GAD67 was 49%; in the VC decreases of 19% in GAD65 protein levels and 20% in GAD67 levels were observed. In young and old Fischer 344 animals, the relative ratios between GAD65 and GAD67 protein levels in the IC, AC and VC followed a very similar pattern as that seen in young and old Long-Evans rats. However, due to the limited number of animals, the data did not show statistically significant differences ($P<0.05$ only when GAD65 or 67 protein levels in the IC were compared with the levels in the AC and VC cortices, Bonferroni`s test).

DISCUSSION

Our results demonstrate significant age-related changes in GAD65 and 67 immunoreactivity in the inferior colliculus and auditory cortex of the rat auditory system, which comprise decreases in the number and optical density of GAD65 and 67-ir neuronal somas and a decline in the levels of GAD65 and 67 proteins. The obtained data demonstrate a rather uniform character of the changes. First, the age-related changes in both GAD65 and 67 proteins shared very similar patterns. Second, the results obtained with immunohistochemical techniques were in good agreement with the results acquired with western blotting. Third,

similar tendencies in the changes were present in both the IC and AC. In summary, the decrease in GAD expression with aging occurs similarly at both the subcortical and cortical levels of the rat central auditory system and involves both GAD isoforms.

The overall reductions in the levels of GAD65 and 67 proteins reported in the present work are in accordance with previous publications that reported an age-related decline in GABA and GAD levels in the IC and AC. In the IC, the number of GABA immunoreactive cells was described to be decreased in the central nucleus of the IC (CIC) in old rats. This decrease was associated with a decrease in enzymatic activity, the levels of GABA and the release of GABA (Caspary et al., 1990; Raza et al, 1994, Guttierez et al, 1994; Caspary et al, 1995). No changes in the optical density of GABA or GAD stained cells in the IC with aging have yet been reported, but such a decline, comparable to our results, was observed in the auditory cortex of old Fischer 344/Brown Norway F1 hybrid rats (Ling et al., 2005). In addition, the authors also reported a decline in GAD65 and 67 mRNA labeling in the auditory cortex, consistent with the decreased optical density of GAD67-ir neurons. No cortical layer specificity for the reported changes was found in their study, which is in agreement with our results. A decline in the optical density of neuronal somas may reflect either a decrease in the expression of the protein or changes in the volume of the cell (for example, Krzywkowski et al., 1995). Since there were no significant changes in the average volume of GAD-ir somas in the present work, the results support the diminished expression of the proteins in this case. This is in agreement with the decreased levels of GAD proteins found in our western blotting analysis.

The observed decreased number of GAD-ir (GAD65 and GAD67) neurons in the IC and AC in the present paper cannot be explained simply by a non-selective neuronal loss because in our previous experiments, we did not find any reduction in the total number of neurons in old Long-Evans and Fischer 344 rats on Nissl-stained sections (Ouda et, al., 2008). This

finding is in agreement with the data of other authors, who reported no significant changes accompanying aging in the total number of neurons in the inferior colliculus, hippocampus or cortex in rats (Helfert et al., 1999; Poe et al., 2001; Merrill et al., 2001; Stanley and Shetty, 2004). In our experiments, the decrease in the optical density of GAD-immunoreactive somas rather suggests the diminished expression of the protein, beyond the level detectable by immunohistochemical methods, as the major reason for the reduced number of GAD-ir neurons.

An interesting finding in our experiments is the striking difference in the regional levels of GAD found with western blotting. The approximately three-fold higher levels of GAD65 and 67 in the IC in comparison with the auditory and visual cortices are in good agreement with previously reported ratios of both isoform levels in the midbrain and thalamus on one side and the neocortex on the other side (Sheikh et al., 1999). The functional implication of these differences remains to be determined. In our immunohistochemical analysis, no significant indication of striking differences between the IC and cortex was observed. Nevertheless, our immunohistochemical method was not designed to evaluate dendritic branching or GAD-ir puncta, which could contain a significant proportion of GAD. The fundamental role of inhibition in the IC is well known, including the strong representation of GABA terminals, which also implies the presence of high GAD levels in the IC (Gerken, 1996; Pollak et al., 2002; Frisina 2001; Batra and Fitzpatrick, 2002; Merchan et al., 2005).

In addition to the Long-Evans strain, a relatively small but representative sample of young and old Fischer 344 rats was examined in the present work. Long-Evans and Fischer 344 represent two rat strains with a variety of morphological, physiological and behavioral differences. For example, similarly to other inbred strains (Wistar, Sprague-Dawley and Dark-Agouti), Fischer 344 rats display large cognitive deficits in different tests of spatial memory in contrast to wild rats and Long-Evans rats (Harker and Whishaw, 2002). A faster and more

pronounced deterioration of hearing function with aging was found in the Fischer 344 strain in comparison with Long-Evans rats, resulting in larger hearing threshold shifts, a diminution of distortion product otoacoustic emissions and a decrease in middle-ear compliance (Popelar et al. 2003, 2006). In addition, the deterioration of the stria vascularis and ligamentum spirale was observed in aged Fischer 344 rats (Buckiova et al., 2006, 2007). Age-related sensory deficits in this strain are also present in visual function due to retinal degeneration (Di Loreto et al, 1994). In spite of this, we observed very similar age-related changes in GAD65 and 67 protein levels and in the immunohistochemical results in Fischer 344 rats and Long-Evans rats. Taking into account the similar effects of aging on GAD levels described in the Fischer 344/Brown Norway hybrid rats (Ling et al., 2005), it might be suggested that age-related changes in GAD proteins in the IC and AC share a uniform tendency, which is not dependent on the rat strain. A similar situation exists in another species, in mice: hearing function in the CBA/CaJ strain of mice deteriorates very slowly as in the Long-Evans strain of rats, whereas the C57BL/6J strain is known to undergo rapid deterioration with early deafness (Parham, 1997, Spongr et al., 1997). In spite of these differences in age-related defects of auditory function, which are mostly caused by pathologies in the inner ear, significant age-related alterations of the inhibitory function in the central auditory system were reported in both strains of mice (Walton et al., 1998, 2002; Felix and Portfors, 2007). Therefore, we may tentatively suggest that the age-related decline in GAD expression in the IC and AC demonstrated in our experiments does not depend primarily on peripheral deafferentation but is, at least partially, of central origin.

The present results are to some extent different from our previous findings concerning age-related changes in parvalbumin immunoreactivity in the IC and AC of Long-Evans and Fischer 344 rats (Ouda et. al, 2008). Specifically, there was no reduction in parvalbumin immunoreactivity in old Long-Evans rats, while there was a pronounced decline in the

number of parvalbumin-ir cells in the AC of old Fischer 344 rats. Since parvalbumin-expressing cells in the central nervous system form a subpopulation of GABA-expressing neurons (Kosaka et al., 1987; Freund and Buzsáki, 1996; Kawaguchi and Kubota, 1998), it could be expected that the changes in parvalbumin and GAD expression with aging would have a more similar character. However, different age-related changes in the immunoreactivity of parvalbumin and the expression of GABA (GAD) were reported, for example, in the rat septum, hippocampus and sensorimotor cortex (Krzywkovski et al., 1995; Vela et al., 2003; Potier et al., 2006; Shi et al., 2006).

Age-related alteration in GABA inhibition was a feature also present in the visual cortex. Our observation of decreased GAD65 and 67 immunoreactivity and protein levels in the visual cortex is in agreement with Hua et al. (2008), who found a reduction in the number of GABA-ir neurons in the primary visual cortex of the cat with aging while no changes in the total number of neurons were observed. In addition, a decreased function of the GABA system was reported in the primary visual cortex of aged monkeys (Schmolesky et al., 2000; Leventhal et al, 2003). Western blot protein analysis in our experiments showed a statistically less pronounced decline in GAD65 and GAD67 levels with aging in the VC compared to the AC, which may signal different GABA-related aging processes in the two cortical sensory areas. A decreased function of the GABA inhibitory system with aging was also documented in the rat hippocampus (Stanley and Shetty, 2004; Shi et al., 2004; Ling et al., 2005), while no reduction in the number of GABA neurons or GAD immunoreactivity was found in the septum, sensorimotor cortex or parietal cortex (Krzywkovski et al., 1995; Poe et al., 2001; Ling et al., 2005; Shi et al., 2006). Therefore, it is evident that aging is not necessarily accompanied with a homogenous decline of GABA levels in all brain regions.

The alteration of GABA-mediated inhibition in the central auditory pathway may have functional implications. The decreased inhibition may result in a broadening of the excitatory

areas and thus in the poorer tuning of the neuronal receptive fields, which is in agreement with existing electrophysiological reports (Palombi and Caspari 1996, Walton et al., 2002, Turner et al., 2005a,b). The reduction of fine-tuned receptive fields results in poorer discrimination of the temporal parameters of sounds (Narayan et al., 2005). The reduced temporal acuity limits the gap detection thresholds and gap duration difference limen, which are belived to be essential for the successful processing of complex sounds including language (Gordon-Salant and Fitzgibbons, 1994; Grose et al., 2006; Gordon-Salant et al., 2007). With aging, the gap detection thresholds significantly increase and gap duration difference limen worsens (Rybalko et al., 2008) These data support the conclusion that the age-related decline of GABA levels could contribute significantly to the deterioration of hearing function associated with aging in mammals, including presbycusis in man.

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Legends to figures:

Fig. 1

Topographical overview of the IC (A), AC and VC (B) and their subdivisions. Drawings adapted from Paxinos and Watson (1998) and Zilles (1995); (A) – bregma -8.8 mm, (B) – bregma -5.0 mm. Par – Parietal cortex; Hip – Hippocampus; PRh – Perirhinal cortex; V1,2 – Visual cortex 1,2; RSD,RSV – Retrosplenial cortex, dorsal and ventral.

Fig. 2

Illustration of GAD67 immunoreactivity in the EIC (A), CIC (B) and in the deep and superficial layers of the AC (C,D) of young Long-Evans rats.

Fig. 3

Results of the quantitative evaluation of GAD65 and 67 immunoreactivity in Long-Evans and Fischer 344 rats. Light columns represent young animals, dark columns old animals. The error bars represent S.E.M. The optical density of neuronal somas is expressed as 100% for young animals.

A, B) Number and optical density of GAD65-immunoreactive neurons in the CIC, EIC, AC and VC of young (n=4) and old (n=4) Long-Evans rats.

C, D) Number and optical density of GAD67-immunoreactive neurons in the CIC, EIC, AC and VC of young (n=5) and old (n=5) Long-Evans rats.

E, F) Number and optical density of GAD67-immunoreactive neurons in the CIC, EIC, AC and VC of young (n=5) and old (n=3) Fischer 344 rats.

(* - $P<0.05$, ** - $P<0.01$)

Fig. 4

Results of western blot GAD65 and GAD67 protein analysis in young (6 for Long-Evans and 2 for Fischer 344) and old (6 for Long-Evans and 2 for Fischer 344) animals of both examined strains. The error bars represent S.E.M.

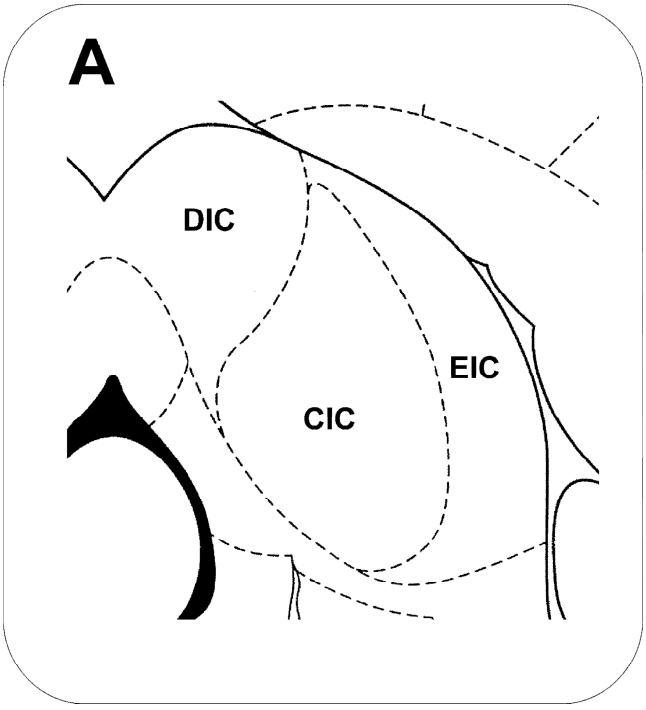
Arbitrary units were calculated as the ratio of the optical density of the examined protein and actin (in scanned films analysed using ImageQuant software).

(* - $P<0.05$, ** - $P<0.01$)

Fig. 5

Representative films of the western blot analysis of GAD 65 (left panel) and 67 (right panels) protein isoforms developed with the enhanced luminiscence method. Actin, used as an internal control, was detected at a position corresponding to a molecular weight of 42 kDa.

Note the partial non-specific labeling on the left panel (non-labeled upper stripes) using polyclonal antibody (for GAD 65). The abbreviations are: AC - auditory cortex, IC – inferior colliculus, VC - visual cortex, Y - young and O - old animals.



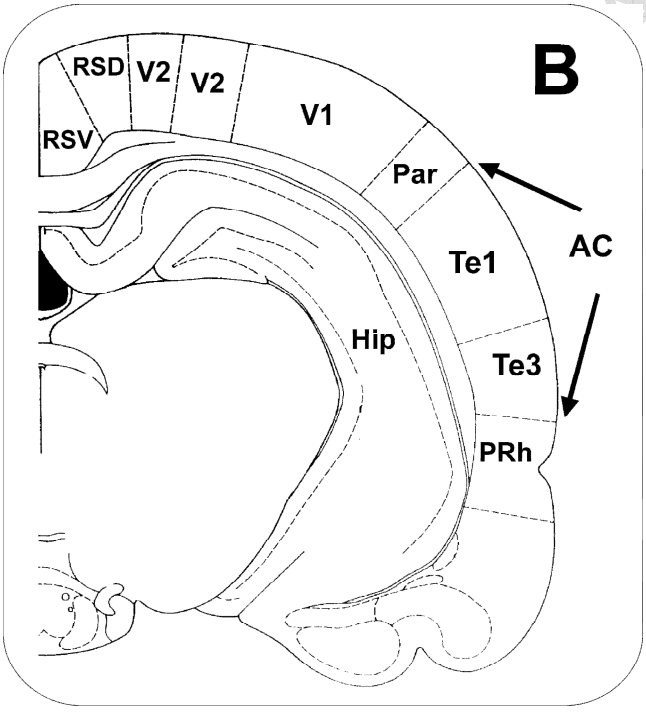


Fig.1

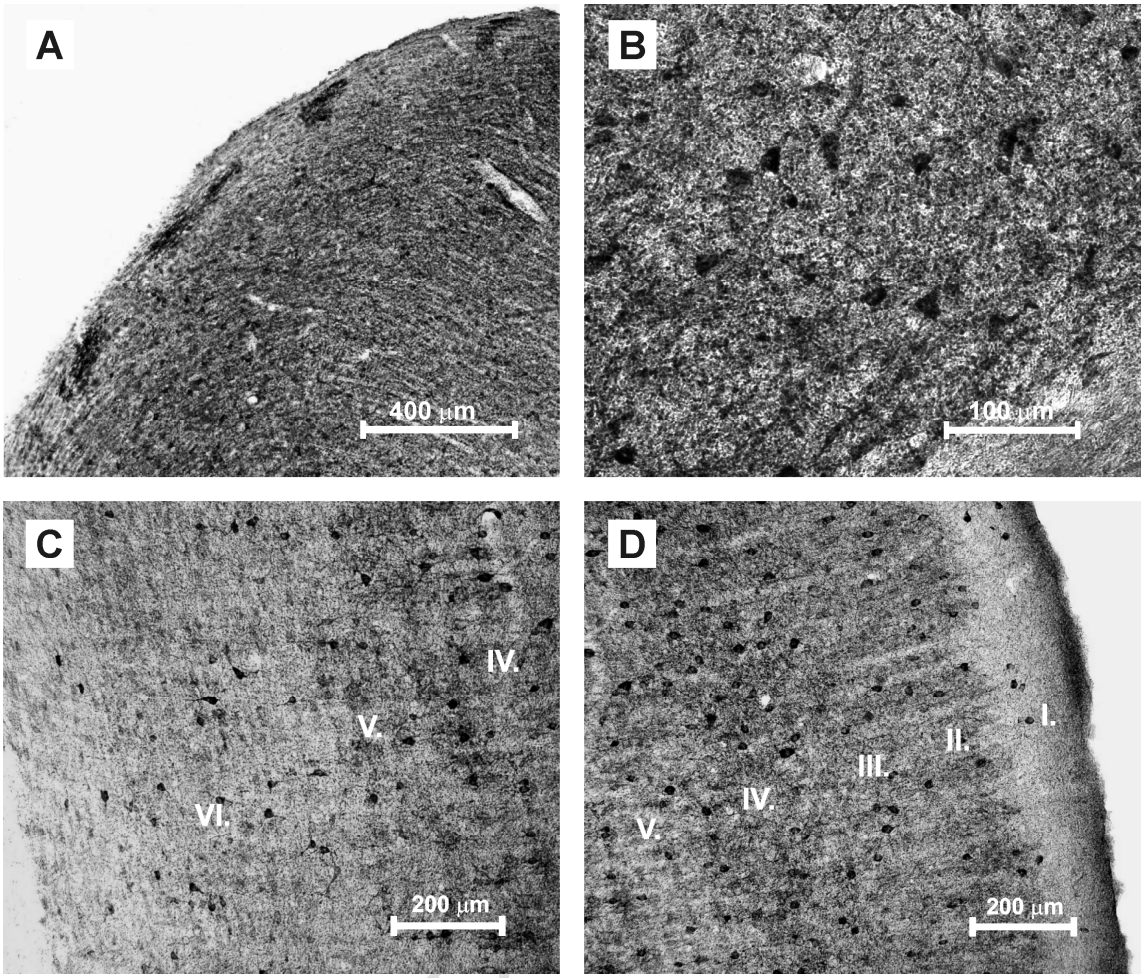


Fig.2

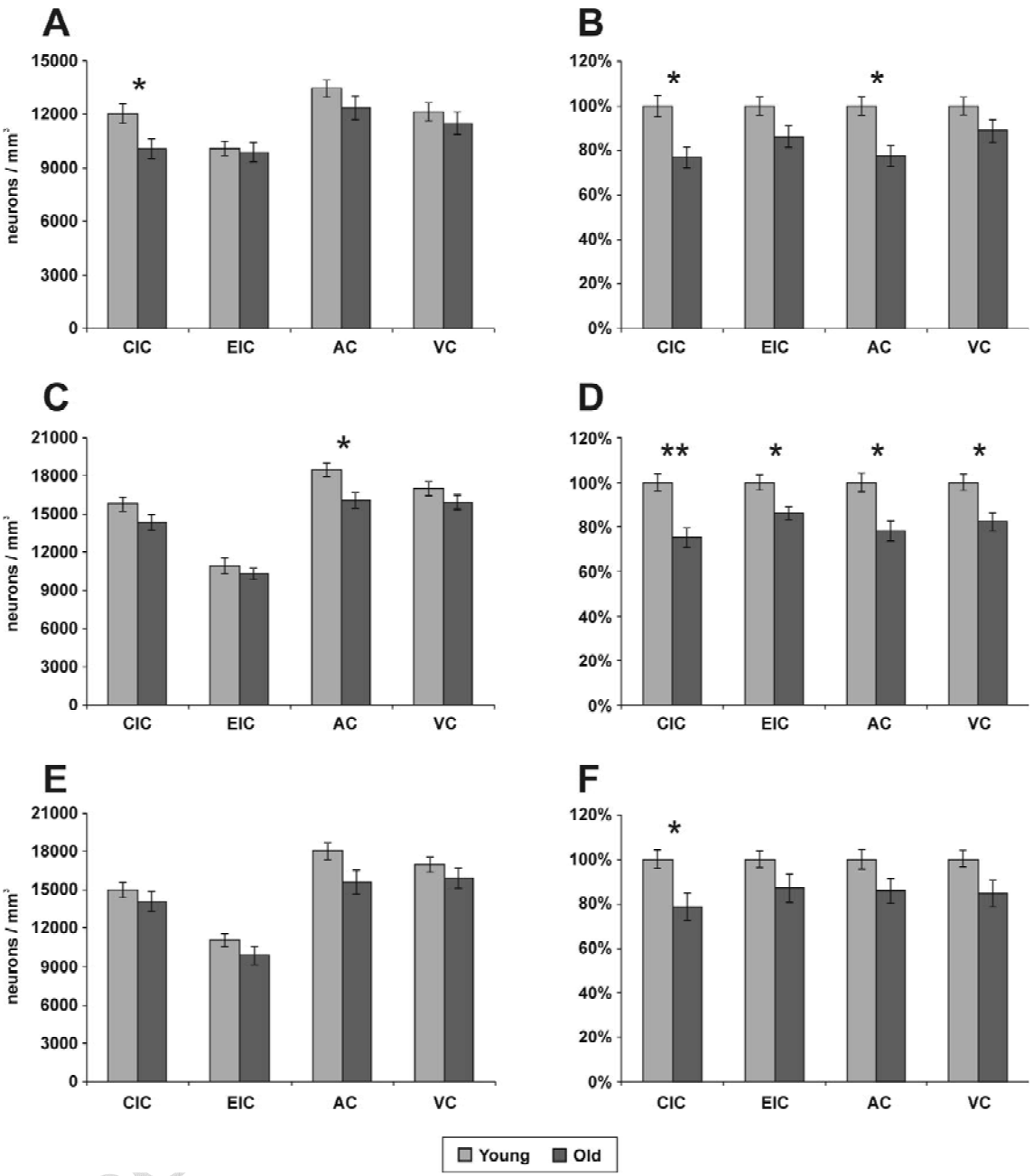

A
B
C
D
E
F
neurons / mm³
15000
12000
9000
6000
3000
0
21000
18000
120%
100%
80%
60%
40%
20%
0%
*
**
CIC
EIC
AC
VC
Young
Old

Fig.3

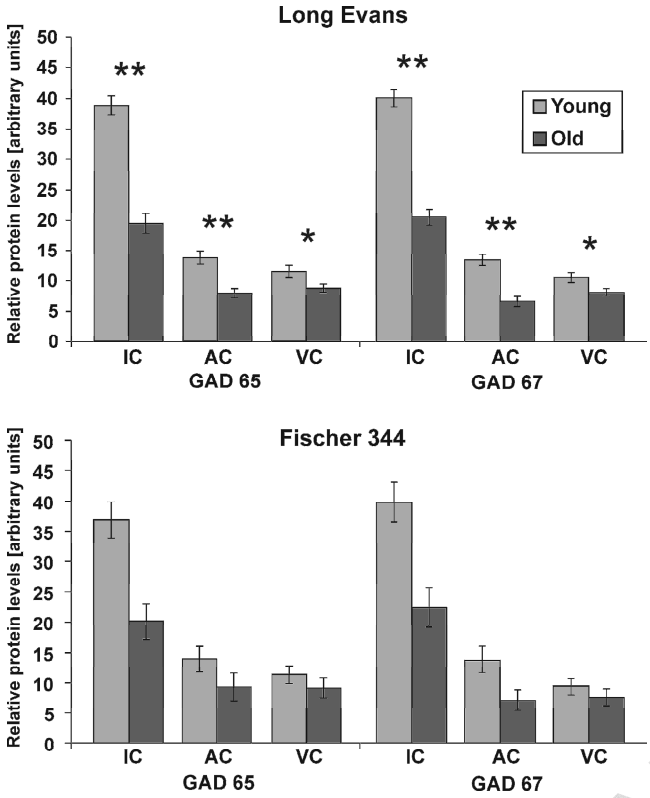


Fig.4

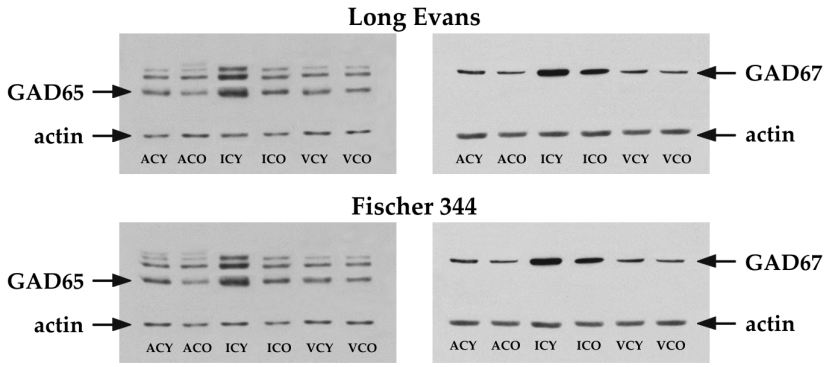


Fig.5