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GABA$_A$ and α-Amino-3-hydroxy-5-methylsoxazole-4-propionate Receptors Are Differentially Affected by Aging in the Rat Hippocampus*

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We have investigated the age-dependent modifications in the expression of eight different subunits of the γ-aminobutyric acid, type A (GABA$_A$) receptor (α1, α2, α3, α5, β2, β3, γ2S, and γ2L) and all four subunits of the α-amino-3-hydroxy-5-methylsoxazole-4-propionate (AMPA) receptor (GluR1–4) in the hippocampus of 24-month-old rats. All aged hippocampi displayed a remarkable increase (aged/adult ratio, 3.53 ± 0.54) in the mRNA levels of the short version of the γ2 subunit in parallel with a similar increase in the γ2 subunit protein (aged/adult ratio, 2.90 ± 0.62). However, this increase was not observed in the mature receptor. On the other hand, the expression of the different α subunit mRNAs increased moderately with aging, displaying a heterogeneous pattern. The most frequent modification consisted in an increase in the expression of the α1 subunit mRNA (aged/adult ratio, 1.26 ± 0.18), in parallel with a similar increase on the α1 protein (aged/adult ratio, 1.27 ± 0.12) and in the α1 incorporated to the assembled GABA$_A$ receptor (tested by immunoprecipitation; aged/adult ratio, = 1.20 ± 0.10). However, in the same hippocampal samples, no major modifications were observed on the expression of the AMPA receptor subunits. As a whole, these results indicated the existence of an increased expression of the GABA$_A$ receptor subunits and a preservation of the AMPA receptor at the hippocampal formation. These modifications could reflect the existence of specific deficiencies (neuronal loss and/or deafferentiation) on the GABAergic system in the aged rats.

Normal aging is associated with memory and/or learning impairments that could reflect modifications at the hippocampal formation (1). The GABA$_A$[1] and AMPA receptors (major fast inhibitory and excitatory receptor complexes, respectively) could be implicated in these alterations (2, 3).

Both neurotransmitter receptors are composed of a high number of subunits in a, probably, pentameric or tetrameric conformation. The GABA$_A$ receptors are formed by the combination of a total of 19 subunits grouped in eight families: α1–6, β1–3, γ1–3, δ, ρ1–3, ϵ, π, and θ (Ref. 5; for a review, see Ref. 4). The AMPA-preferring ionotropic glutamate receptor is composed by four subunits (GluR1–4) displaying different splicing isoforms (for a review, see Ref. 6). This high molecular heterogeneity can generate multiple receptor isotypes, displaying particular physiological and pharmacological properties.

It is known that the sensitivity for benzodiazepines (anxiolytic/hypnotic drugs that interact to the GABA$_A$ receptor) increase during aging in humans and in rodents (7). Previous work from our group has demonstrated the existence of aging-associated modifications in both the pharmacological properties and the molecular composition of the GABA$_A$ receptors in rat hippocampus (8–10). These changes could reflect a sensitization process of the GABA$_A$ receptor (see also Ref. 11). However, the age-dependent modifications on the expression of the different subunits of the GABA$_A$ receptor are currently unknown. This lack of knowledge is probably due to both the high number of subunits expressed at the hippocampal formation and the heterogeneity of the aging process. On the other hand, the excitatory glutamate receptors, especially AMPA-preferring glutamate receptors, seem to be less vulnerable to normal aging, as revealed by the absence of modifications on its binding properties (12–14). Therefore, the hippocampal GABAergic system seems to be preferentially affected in the aged rats (Ref. 15 and references therein).

Aiming at obtaining an extensive and global knowledge of the possible age-dependent alterations in the expression of both GABA$_A$ and AMPA receptors, we have quantified, using reverse transcription (RT)-PCR, the expression of 18 different mRNAs implicated on both the GABAergic system and the AMPA receptor in each hippocampal sample. We are aware that aging also displays anatomical heterogeneity (10, 15, 16), and with our approach, we cannot resolve these modifications. However, we have analyzed the expression of eight GABA$_A$ receptor subunits (α1, α2, α3, α5, β2, β3, γ2S, and γ2L, the most abundantly expressed subunits at the hippocampal formation) and all four AMPA-preferring glutamate receptor subunits (GluR1–4), including their flip and flop variants, together with both GAD isoenzymes in the same hippocampus from adults and 24-month-old rats. Furthermore, using the same samples, we have also quantified the relative abundance of two subunit proteins (α1 and γ2 subunits) of the GABA$_A$ receptor complex that consistently increased during aging. In all, we have obtained a significant amount of information about the

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[1] The abbreviations used are: GABA$_A$, γ-aminobutyric acid, type A; AMPA, α-amino-3-hydroxy-5-methylsoxazole-4-propionate; bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; ANOVA, analysis of variance; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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age-dependent modifications of the expression of different subunits of the two major fast neurotransmitter receptors.

MATERIALS AND METHODS

Hippocampus Isolation—Adult (3 months) and aged (24 months) Wistar rats were killed by decapitation, and the hippocampi were rapidly dissected and frozen in liquid N_2. The hippocampi were stored at −80 °C until use.

RNA and Protein Extraction—Total RNA and proteins were extracted using the Tripure™ isolation reagent (Roche Molecular Biochemicals) kit, according to the instructions of the manufacturer. This procedure allows the isolation of total RNA, DNA, and protein fractions from a single sample. The contaminating DNA in the RNA samples was removed by incubation with DNase and confirmed by PCR analysis of total RNA samples prior to RT. After isolation, the integrity of the RNA samples were assessed by agarose gel electrophoresis. The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol-precipitated aliquots of the samples. The recovery of RNA was similar for both young and old hippocampi (not shown).

To analyze the protein fraction, the protein pellets obtained using the Tripure™ isolation reagent, from adult and aged hippocampus, were resuspended in 1% SDS, 10 mM Tris-HCl, pH 7.4. The total recovery and integrity of these fractions were determined as (17) and SDS-polyacrylamide gel electrophoresis.

Competitive RT-PCR—RT was performed in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, 1 mM random hexamers, 50 units of ribonuclease inhibitor, and 100 units of AMV-RT (Roche Molecular Biochemicals) in a final volume of 20 μl and 1 μg of total RNA as template. After RT, samples were treated with RNase, and free nucleotides were eliminated using GlassMAX spin cartridges (Life Technologies, Inc.). As control of the efficiency in the RT, samples from adult and aged hippocampi were reverse-transcribed as described above but in presence of 2 μM digoxigenin-dUTP, and the purified cDNA was dotted on Nylon membranes and developed (see below). The films were scanned and the adult and aged samples were compared. The results (aged/adult ratio, 1.10 ± 0.40, n = 11) demonstrated a similar RT efficiency in both ages.

Competitive RT-PCR was performed basically as described (18). Briefly, aliquots of hippocampal cDNA (100 ng/tube) and known quantities of internal standards, corresponding to the α1, α2, α3, α5, β2, β3, γ2S, and γ2L $GABA_A$ receptor subunits, were mixed in different tubes with increasing amount of internal standards. Each internal standard consisted of the same sequence amplified in the native subunit, but modified by the inclusion of a *Bgl*II restriction site, cloned into pGEM-1 plasmids (kind gifts from Dr. Dennis R. Grayson) (19). For each $GABA_A$ receptor subunit, the range of internal standard quantities was established in control experiments using adult hippocampus. For each subunit, the range was as follows: 10–1000 fg for α1, α3, α5, β2, and β3; 100–1000 fg for α2; and 10–10,000 fg for γ2 (S or L isoforms).

The PCR was performed in a final volume of 50 μl, using *Taq* polymerase, 2.5 units, in the buffer supplied by the manufacturer (Ecogen); 1.5, 2, or 3 mM $MgCl_2$, for the α, β, or γ subunits, respectively, including 1 μM each 5′ (sense) and 3′ (antisense) of the respective primers pairs (20); and 50 μM dNTPs with the addition of 2 μM digoxigenin-dUTP (Roche Molecular Biochemicals). Internal standards and cDNA were heat-denatured for 5 min at 94 °C, and tubes were kept on ice until ready for PCR. The PCR was performed with 30 cycles consisting of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 50 s, followed by a final elongation period of 5 min at 72 °C in a Techne Progene Thermal Cycler. The PCR products (5–10 μl) were digested overnight with 10 units of *Bgl*II and separated on a 1.7% agarose gel in 0.5× Tris-borate-EDTA buffer. After electrophoresis, the PCR products were transferred onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) using a vacuum blotting system, VacuGene™ XL (Amersham Pharmacia Biotech) for 1 h at 50 mbar of pressure. The nylon membranes were blocked, incubated with an anti-digoxigenine antibody conjugated with peroxidase (dilution, 1/20,000; Roche Molecular Biochemicals), washed with Tween 0.1% in phosphate-buffered saline, and processed for chemiluminescence detection using the ECL-plus (Amersham Pharmacia Biotech) following the instructions of the manufacturer. Films were developed and scanned with a laser densitometer (Molecular Dynamics, model 300 A). Bands for both native cDNA (uncut band) and internal standard (cut band) were analyzed, and the data are the ratio between internal standard and native cDNA (see Fig. 1). All subunits were determined in at least duplicate, and for each experiment, a minimum of one adult and one aged samples were processed and analyzed in parallel.

Control experiments for the efficiency of the transference, second antibody dilution, and film exposure were performed. In all cases, we found a linear correlation between the amount of PCR products and the detected absorbance on the films (not shown).

Quantification of the AMPA Subunits and Flip/Flop Relative Proportions—The same samples used for the PCR amplification of the $GABA_A$ receptor subunits, from adult and aged rat hippocampus, were amplified using primers common to GluR1–4 (sense primer, CCTTTGGCCTATGAGATCTGGATGTG; antisense primer, TCGTACCACCATTTGTTTTTCA) with 35 PCR cycles, as described (20), but including 2 μM digoxigenin-dUTP. The PCR products, 750 bp, (10 μl per subunit) were digested by *Bgl*I, *Ban*II, *Eco*47III, or *Eco*RI restriction enzymes, which specifically cut the GluR1 (300 and 449 bp), GluR2 (478 and 271 bp), GluR3 (359 and 396 bp), or GluR4 (411 and 338) PCR fragments, respectively. The restriction products were separated on a 1.7% agarose gel and transferred to Hybond membranes, and the films were generated and processed as described above. Quantification was performed for each electrophoresis lane, corresponding to a subunit-specific digestion, by summing the absorbance values of cut and uncut bands and normalizing to 100%. Thus, the percentage of the cut bands corresponds to the proportion of GluR1–4 subunits present in the PCR-amplified product.

The total amount of all four AMPA receptor subunits was estimated by quantifying the total, undigested PCR products. For comparative proposes, the results were normalized by the abundance of the β-actin (see below).

Flip/flop proportion of the GluR1–4 subunits was quantified using the product of the first PCR (see above) as a template for a second PCR (in the presence of 2 μM digoxigenin-dUTP). Specific sense primers for either GluR1, -2, -3, or -4 and the common antisense primer was that used for the first amplification. The PCR products (632, 639, 628, and 630 bp, corresponding to GluR1, -2, -3 and -4, respectively (both flip and flop in all cases)) were then cut with subunit specific enzymes (*Bgl*I for GluR1 flip and GluR2 flop, and *Hpa*I for GluR3 flop and GluR4 flop (20, 21)) and processed as described above.

GAD65/67 and β-Actin Quantification—Both isoforms of the glutamic acid decarboxylase (GAD65 and GAD67) were amplified from the same cDNA samples as for $GABA_A$, and AMPA receptors. For the detection of GAD65 and GAD67 mRNAs, the following set of specific primers were used, from 5′ to 3′: GAD65 sense, TCTTTTCTCCTGGTGGTGCC (position 713); GAD65 antisense, CCCCAAGCAGCATCCACAT (position 1085); GAD67 sense, TACGGGGTTCGCACAGGTC (position 713); GAD67 antisense, CCCCAAGCAGCATCCACAT (position 1159). The same antisense primer was used for the amplification of both GAD65 and GAD67. This primer had one mismatch with the sequence of the GAD67, as indicated by the underlined base (21). The PCR products were processed as described above. The results were normalized by the expression of a housekeeper gene, β-actin.

The β-actin was amplified using the specific primer pairs, from 5′ to 3′: sense, CGGAACCGCTCATTGCC; antisense, ACCCACACTGTGCCCATCTA. PCR was performed in the presence of 2 μM digoxigenin-dUTP.

In all cases, the GAD65, GAD67, AMPA, and β-actin were amplified, electrophoresed, and processed in parallel. In addition, samples for adult and aged rats were also processed and analyzed in parallel. For quantification, three different cDNA dilutions were used, and 20, 25, and 30 cycles of PCR were run in order to avoid the problem of band saturation.

Membrane Preparation and Receptor Solubilization—Hippocampal membranes from 3- and 24-month-old Wistar rats were prepared by ultracentrifugation at 100,000 × g as described elsewhere (8, 22, 23 and ref. therein) in presence of protease inhibitors: 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 50 μg/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin.

The $GABA_A$ receptor was solubilized at 1 mg of protein/ml, 4 °C for 60 min, with 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) CHAPS, 140 mM NaCl, and 10 mM Tris-HCl, pH 7.5, containing the same protease inhibitors as above. After centrifugation at 100.000 × g, for 60 min at 4 °C, the supernatant was collected. Previous work has demonstrated the absence of subunit redistribution using this solubilization conditions (24).

Antibody Purification and Immunoprecipitations—When needed, the anti-α1 and anti-γ2 antibodies (24, 25) were purified through peptide affinity columns. The peptides were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). One ml of the different antisera (diluted 1/5 in phosphate-buffered saline) were recirculated overnight at 4 °C in the corresponding column (1 ml). After washing with 150 ml phosphate-buffered saline, the antibodies were eluted with 50

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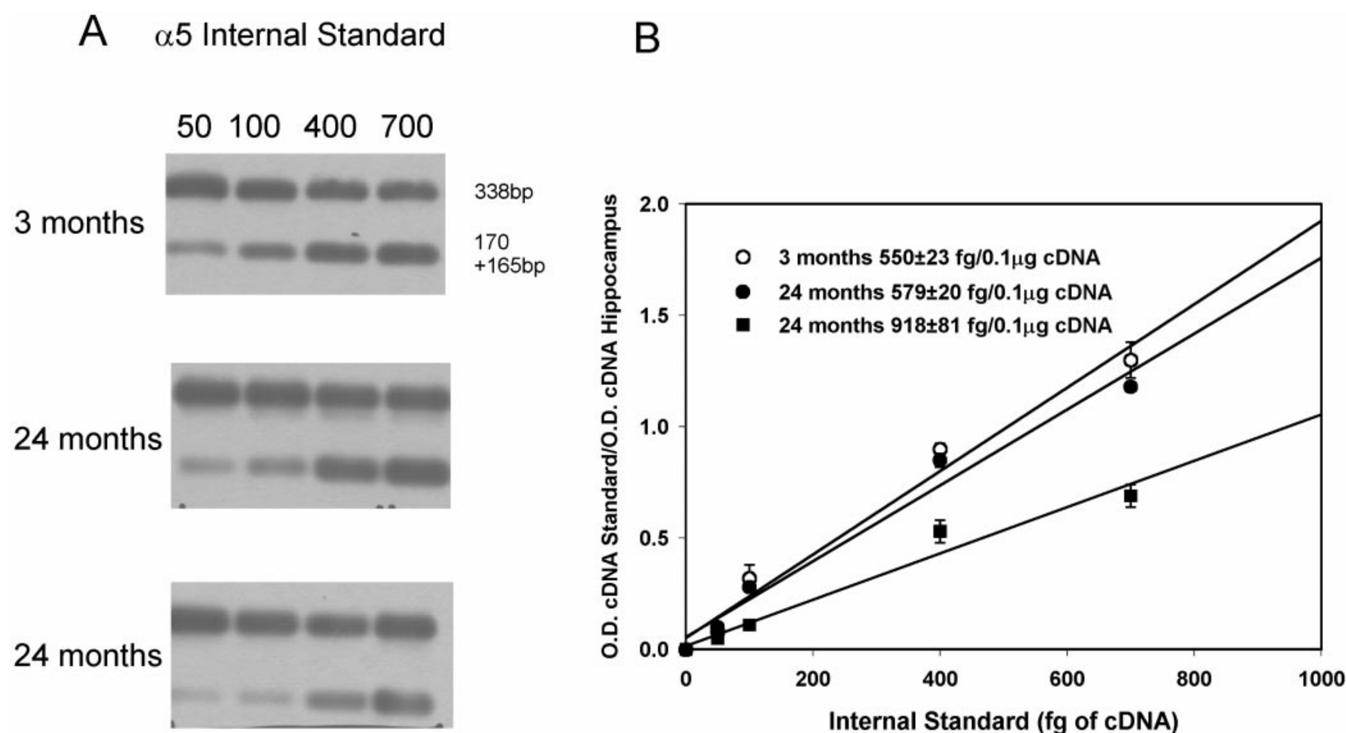

FIG. 1. **Representative quantification of the GABA$_A$ receptor α5 subunit mRNA levels in one adult and two different aged hippocampi.** *A,* representative films generated after the amplification of a constant amount of cDNA (100 ng) in presence of four different amounts of α5 internal standard. The PCR products were digested by *Bgl*II and electrophoresed in agarose gels. The higher molecular size band (338 bp) corresponded to native cDNA, and the smaller product corresponded to the internal standard products (170 + 165 bp). *B,* the films (shown in *A*) were analyzed by densitometry and the ratio of internal standard (absorbance)/sample cDNA (absorbance) was plotted *versus* the amount of internal standard used. The results are expressed as mean ± S.D. of triplicate measurements. The point of equivalence (also shown in the figure) was calculated after linear regression of the curves.

mM glycine-HCl, pH 2.3, and the fractions (0.5 ml) were neutralized by 1 M Tris, pH 11, pooled, and dialyzed against 1 liter of phosphate-buffered saline overnight at 4 °C.

Prior to utilization, saturating amounts (not shown) of the different antibodies were adsorbed to 50 μl of a suspension of protein A-Sepharose (10% (w/v) in solubilization buffer; see also Refs. 24 and 25). The immunoprecipitations were done as described (24, 25).

The immunoprecipitation was quantified by determining the binding activity of 10 nM [^{3}H]flumazenil (total benzodiazepine receptors) in both pellets and the final supernatants. The binding assays were done essentially as described previously (23).

Immunoaffinity Chromatography—It is known that the γ2 subunit displays an anomalous electrophoretic behavior in SDS-polyacrylamide gel electrophoresis (26, 27), and it is difficult to detect in Western blots (28). Thus, the γ2 subunit from adult and aged hippocampus was first immunopurified through anti-γ2 affinity columns (our anti-γ2 antibody was made against a peptide of the N-terminal domain of the protein, and thus, it recognizes both short and long isoforms). The anti-γ2 immunoaffinity columns were synthesized as described (25). Fab fragments of the purified anti-γ2 antibodies were used in order to avoid any possible interference with the IgG heavy chain (55 kDa). The Fab fragments were prepared using papain-agarose (Pierce) as recommended by the manufacturer. The Fab immunoaffinity columns were synthesized using CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech).

For immunopurification of the γ2 subunits, the protein fractions (containing 1% of SDS, see above) were diluted 1/5 with 1% (w/v) Triton X-100, 140 mM NaCl, 10 mM Tris-HCl, pH 7.4, and aliquots of 50, 100, and 200 μg of proteins were applied to 200 μl of affinity column. After overnight adsorption (at 4 °C), the columns were extensively washed and eluted with 2% SDS in 10 mM Tris-HCl, pH 7.4. The eluted receptor was electrophoresed and processed for immunoblot (25). For each determination, one adult and one aged hippocampus were processed in parallel. Importantly, the immunopurification approach was quantitative, because after a first round of immunopurification, no immunoreaction product (M_r 43,000 peptide) (24) was detected in a second round of anti-γ2 immunopurification (see Fig. 3*A*). For the comparison between adult and aged rats, three different aliquots were immunopurified and analyzed in parallel.

Other Methods—Immunoblots, protein determination, and SDS-polyacrylamide gel electrophoresis were done as described elsewhere (24, 25). The statistical analysis of the data were performed using one-way ANOVA or multifactor ANOVA and the Tukey post hoc multiple comparisons test.

RESULTS

Expression of the GABA$_A$ Receptor Subunit mRNAs—The expression of different subunits of the GABA$_A$ receptor was quantified by competitive RT-PCR using cDNAs from hippocampus of 3-month-old (adult) and 24-month-old (aged) rats. Fig. 1 shows a representative experiment of the competitive RT-PCR analysis of the α5 subunit mRNA from one adult and two aged rats. This experiment illustrates the linearity of the competitive RT-PCR (in the range selected for each subunit of the GABA$_A$ receptor; see under "Materials and Methods"), the experimental variability of triplicate measurements, and also the differences between the adult and the different aged samples. Thus, using this approach, we have quantified the expression of eight subunits of the GABA$_A$ receptor complex (α1, α2, α3, α5, γ2S, γ2L, β2, and β3 subunits; the major subunits expressed at the hippocampal formation) (29, 30) in a total population of four adults and five aged rats. As shown, Fig. 2*A,* in the adult and aged rats, the expression of the different GABA$_A$ receptor subunits agrees with previous reports using *in situ* hybridization (29). It is interesting to note the low inter-individual variation found in the four adults tested. The coefficients of variation for each subunit (calculated as SD/mean) were 0.13, 0.11, 0.26, 0.16, 0.39, 0.24, 0.27, and 0.22 for α1, α2, α3, α5, γ2 s, γ2L, β2, and β3, respectively (mean, 0.22).

By comparing all subunits in both ages, we observed a significant increase in the aged population (multifactor ANOVA F (1,71) = 59.66, p = 0.00001) and also significant differences between subunits and ages (subunit × age, multifactor ANOVA

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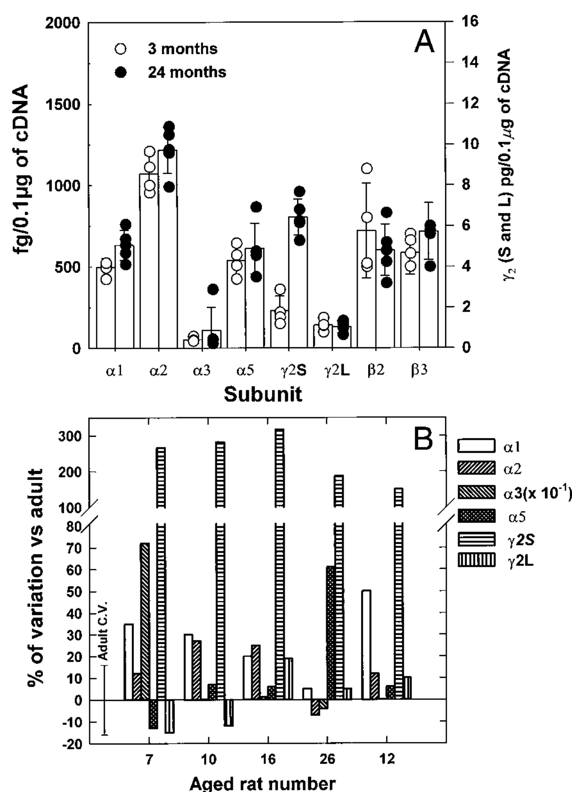

FIG. 2. **Age-dependent modification in the expression of the hippocampal GABA$_A$ receptor subunits.** *A,* shown are the quantitative levels of the mRNAs coding for eight GABA$_A$ receptor subunits from four adult (*open circles*) and five aged (*closed circles*) rat hippocampi. The results are shown individually and as mean ± SD (*bars*). Note the difference in scale for both axis. *B,* the percentage of variation (in relation to the adult values) of the α and γ2 (S and L version) subunits was determined individually for each analyzed rat. The coefficient of variation of the adults for these subunits was also indicated in the figure.

F (7,71) = 55.36, p = 0.00001; Tukey $p < 0.05$). When the expression of the total mRNA levels for the different subunit families (*i.e.* α1 + α2 + α3 + α5, β2 + β3, and γ2S + L) was analyzed, a significant increase in the aged rats was observed for the α and γ2 subunits (Table I). The expression of the GABA$_A$ receptor from aged hippocampus showed an important high interindividual variability. The coefficients of variation, calculated as above, for the expression of the different subunits in the aged rats were 0.20, 0.12, 1.2, 0.26, 0.14, 0.28, 0.26, and 0.24 for α1, α 2, α3, α5, γ2 s, γ2L, β2, and β3, respectively (mean, 0.34). This high variability is likely due to the aging process and not an artifact because of the competitive RT-PCR (see Fig. 1). Thus, we also analyzed individually the percentage of variation (*versus* adult) in the expression of the α and γ subunits. As shown in Fig. 2*B,* the expression of these GABA$_A$ receptor subunits showed a clear tendency to increase during aging. In fact, the expression of the γ2S subunit increased notably in the hippocampus from all five tested rats (see also Fig. 4, in which a new aged sample was analyzed) (ranging from 190 to 320% of the adult values; mean, 253.8 ± 53.9%; ANOVA F (1,7) = 69.33, p = 0.001; Tukey $p < 0.01$) with no modifications in the expression of the long splicing version of this subunit. Consequently, the S/L ratio (1.81 ± 0.7 *versus* 6.3 ± 1.9 for adult and aged rats, respectively) also significantly increased in the aged hippocampi (F (1,7) = 21.9, p = 0.002; Tukey $p < 0.05$).

On the other hand, the age-dependent modifications in the expression of the different α subunits were highly heterogeneous (see Fig. 2). The most common pattern of variation in the

TABLE I
Age-dependent modifications in the total mRNA content of the different subunit families of the hippocampal GABA$_A$ receptor

The total expression of the different subunit families was calculated as the sum of the mRNA content of the different subunits tested (*e.g.* α1 + α2 + α3 + α5; β2 + β3; γ2s + γ2L). The results are mean ± S.D. of four and five different experiments for adult and aged rats, respectively.

Age	Total mRNA (fg/0.1 µg cDNA)		
	α subunits	β subunits	γ2 subunits
3 months	2157 ± 136	1310 ± 158	3717 ± 917
24 months	2563 ± 107[a] (+19.0 ± 5.1%)	1318 ± 236	9284 ± 1237[b] (+250 ± 48%)

[a] ANOVA F (1,7) = 24.94, p = 0.002; Tukey $p < 0.05$.
[b] ANOVA F (1,7) = 55.7 p = 0.0001; Tukey $p < 0.01$.

aged rats was an increase in the α1 subunit mRNA (four of five animals; ranging from 17 to 50%; mean, 32.5 ± 14.7%; significant difference from adult, ANOVA F (1,6) = 9.59, p = 0.02; Tukey $p < 0.05$). It is noteworthy that the single aged hippocampus displaying no differences in the α1 level, rat 26, was also the only aged rat showing a notable increase in the expression of the α5 subunit (see Figs. 1 and 2; 61.1% over the adult value). It is also interesting that in most cases (rats 7, 10, and 16), the increment in the α1 subunit was also accompanied by an increase in other α subunit(s) (see Fig. 2*B*), such as α2 (rats 10 and 16; 28 and 25% respectively) or α3 (rat 7; 700%).

Expression of the γ2 and α1 Subunit Proteins—We next compared the expression of the proteins corresponding to α1 and γ2 subunits, using the protein fractions obtained from the same hippocampal samples as above (see under "Materials and Methods").

Fig. 3*C* shows the immunoreaction product corresponding to the immunopurified γ2 subunit (43 kDa), which clearly increased in the aged samples (compare *lanes 1–3* with *4–6*). The difference between both ages was calculated as the aged/adult ratio after the densitometrical analysis of the films. As shown in Fig. 4*A,* there was a remarkable and significant increase in the expression of the γ2 subunit protein (aged/adult ratio, 2.90 ± 0.62, n = 6, ranging from 1.7 to 3.3; ANOVA F (1,8) = 26.43, p = 0.0009; Tukey $p < 0.01$). This increase was similar to that detected at the mRNA level (γ2S+L, aged/adult ratio, 2.40 ± 0.40, n = 6, Fig. 4*A*). When these results were analyzed individually (Fig. 4*B*), the alterations in mRNA and protein levels for the GABA$_A$ receptor γ2 subunit were found to vary in parallel.

The α1 subunit was directly quantified by Western blots. The anti-α1 antibody immunoreacted with a M_r 51,000 band (Fig. 3*B,* corresponding to the α1 subunit; see also Refs. 24 and 25), and the immunoreaction product increased with the amount of protein loaded in the gel. As also shown (Fig. 3*B*), no signal was detected in absence of antibody or by preincubation of the anti-α1 antibody with the corresponding peptide (data not shown, but see Ref. 25). As shown (Fig. 3*D*), in the sample from adults, the anti-α1 antibody immunoreacted with a major 51-kDa band and with a faint band of 53–54 kDa. This minor component was not observed using total proteins from the adult rat cortex (Fig. 3, *B* and *D,* CTX), membranes isolated from different rat brain areas, including hippocampus (31), or in immunopurified receptors (see Ref. 25; also see Ref. 32). Interestingly, in all aged hippocampi, this 53–54-kDa band was strongly and consistently recognized by the anti-α1 antibody (see Fig. 3*D*). Furthermore, this 53–54 kDa band was observed in the aged rats even using purified (through peptide columns) anti-α1 antibody (not shown). In the aged samples, the intensity of this immunoreaction product parallels that of the 51-kDa band. In fact, the 53–54-kDa band represented the 28.0 ± 5.8%, n = 6, of the 51 kDa. Although this band could be a

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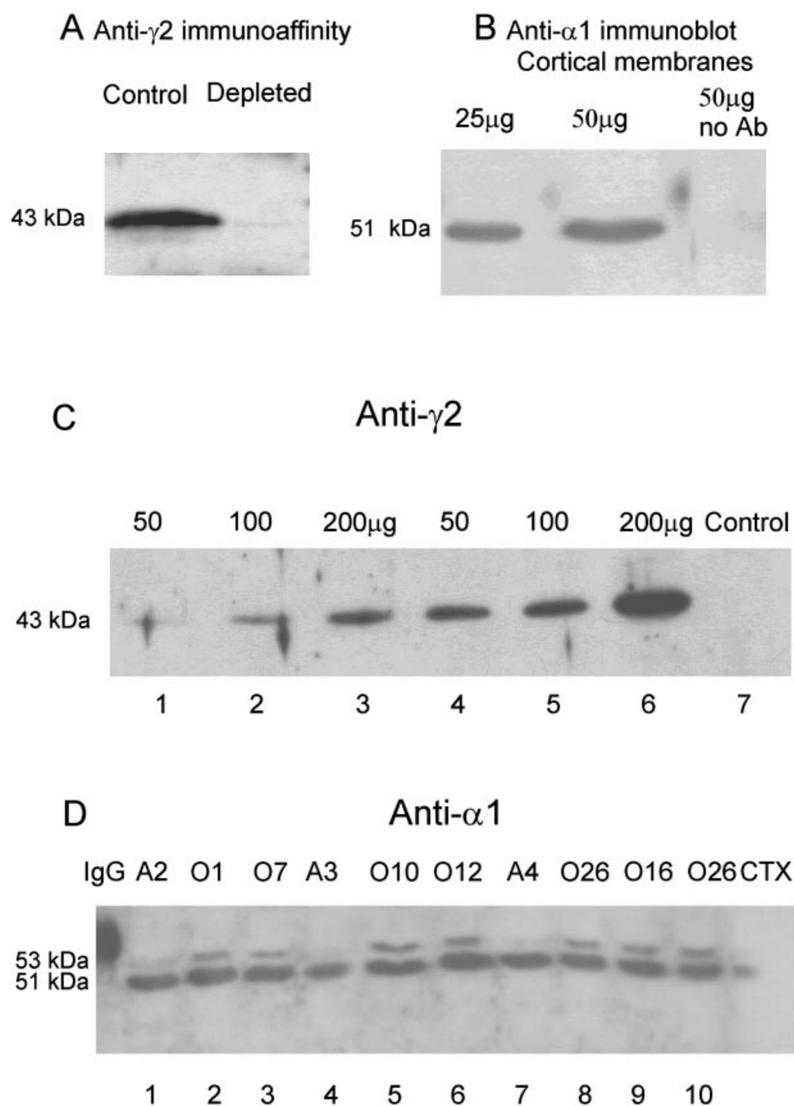

FIG. 3. **The protein levels of both γ2 and α1 subunits increased in the aged hippocampus.** The levels of γ2 and α1 subunit proteins were determined using specific antibodies by immunoaffinity or immunoblot experiments. *A,* control experiments showing that the anti-γ2 immunoaffinity columns retained most, if not all, of the γ2 subunit in single round of immunopurification. The protein fraction, obtained after RNA isolation of an adult hippocampus, was immunopurified through 200 μl of anti-γ2 affinity column. The supernatant of this purification was used in a second round of incubation using the same amount of anti-γ2 affinity column. Both columns were washed and eluted with 2% SDS. The presence of the γ2 subunit was analyzed by Western blot using a 1/5000 dilution of anti-γ2 antibody. As shown, a clear 43-kDa band was detected in the first immunopurification (*control*), whereas no reaction products were detected after the second immunopurification (*depleted*). *B,* Western blot analysis of the α1 subunit using cortical membranes. Two different amounts of membranes were subjected to Western blot with a 1/1000 dilution of anti-α1 antibody. This antibody recognized a band of 51 kDa (α1), the immunoreaction product increased in function of the protein loaded, and this product is absent in absence of primary antibody or in presence of anti-α1 plus peptide (not shown). *C,* representative experiment of the anti-γ2 (200-μl affinity column) immunopurification procedure using three different amount of proteins (50, 100, and 200 μg) from adult (*lanes 1–3*) and aged (*lanes 4–6*) hippocampus processed in parallel. Note the clear increase of the anti-γ2 immunoreaction products in the aged sample. A control of the anti-γ2 immunoaffinity column, in the absence of sample (*lane 7*), was also included. *D,* Western blot analysis of the α1 content of a fixed amount of protein (15 μg) from three adults (A2, A3, and A4: *lanes 1, 4,* and *7,* respectively) and 7 aged samples (O1, O7, O10, O12, O26, O16, and O26: *lanes 2, 3, 5, 6, 8, 9,* and *10,* respectively). This experiment was repeated three times, in a similar configuration, but testing all four control hippocampus. Note the clear difference between the adult and aged samples on the pattern recognized by anti-α1 antibody.

different glycosylation form of the α1 subunit (32), the precise nature of this band is currently unknown and was not considered for quantification.

The quantification of these experiments, shown in Fig. 5*A,* indicated the existence of a moderate (aged/adult ratio, 1.27 ± 0.12, ranging from 1.05 to 1.40, $n = 6$) but significant (ANOVA F (1,10) = 4.9, $p = 0.04$; Tukey $p < 0.05$) increase in the total content of α1 subunit in the aged hippocampus. Furthermore, the age-dependent increase in the expression of the α1 subunit also correlates with the variation in the mRNA levels (aged/adult ratio, 1.26 ± 0.18, $n = 5$). When the data were analyzed individually (see Fig. 5*B*), variation on the mRNA levels was also reflected by a similar modification at the protein level (*e.g.* rat 26).

Age-dependent Modifications of the Native GABA$_A$ Receptor Complex—We also tested the effect of aging in the expression of these two subunits, γ2 and α1, assembled in native GABA$_A$ receptors. To this end, we analyzed the [^{3}H]flumazenil binding activity (10 nM) immunoprecipitated by anti-γ2 and anti-α1 antibodies from hippocampal membranes of a new population of four adult and four aged hippocampi. The results (see Table II) demonstrated that both the [^{3}H]flumazenil total binding activity and the anti-γ2 immunoprecipitated binding activity (expressed in pmol/mg of solubilized protein) significantly de-

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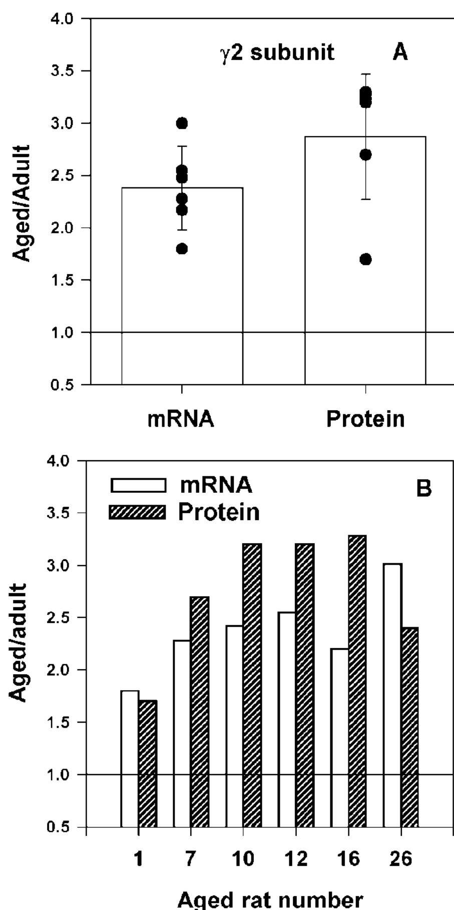

FIG. 4. **Age-related alterations on the expression of the γ2 subunit.** *A,* shown are the age-dependent variations, expressed as aged/adult ratio, on γ2 subunit mRNA (short + long isoforms) and protein. The mRNA data were taken from Fig. 2 and protein variation were calculated from experiments similar to that shown in Fig. 3*C*. Data are presented individually (*closed circles*) or as mean ± SD (*open bars*). *B,* the variations on the aged population (aged/adult ratio) in both the γ2 mRNA (*open columns*) and protein (*hatched columns*) were represented individually for each tested rat.

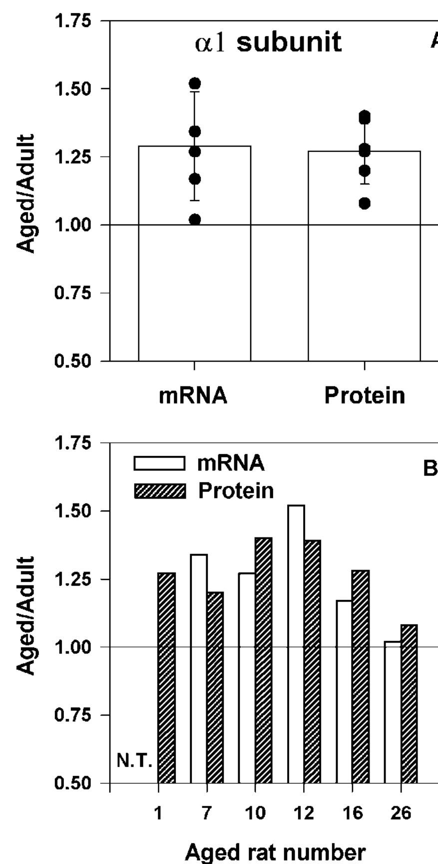

FIG. 5. **Age-related alteration on the expression of the α1 subunit.** *A,* shown is the variation (aged/adult ratio) of the mRNA and protein corresponding to the α1 subunit (see legend of Fig. 4 for more details). Data are presented individually (*closed circles*) or as mean ± SD (*open bars*). *B,* the variations on the aged population (aged/adult ratio) in both the α1 mRNA (*open columns*) and protein (*hatched columns*) were represented individually for each tested rat. *N.T.,* not tested.

creased in all four aged hippocampi tested. On the other hand, after two rounds of incubation, the anti-γ2 antibody immunoprecipitated most, if not all, the [^{3}H]flumazenil binding activity in both ages (90.9 ± 7.2% and 91.5 ± 3.1% for adult and aged hippocampi, respectively, see Table II) (see Ref. 23). As also shown (Table II), there was a slight (and not statistically significant) decrease on the [^{3}H]flumazenil binding activity immunoprecipitated by the anti-α1 antibody in the aged hippocampus. This decrease was lower than that expected by the reduction of the total [^{3}H]flumazenil binding observed in the same membrane preparation (–23.8 *versus* –8.6% for total and anti-α1 immunoprecipitated binding activity, respectively; see Table II). As a consequence, the proportion of [^{3}H]flumazenil binding activity immunoprecipitated by anti-α1 antibody increased significantly in the aged samples (54.5 ± 3.8 *versus* 65.1 ± 5.6% for adult and aged; aged/adult ratio, 1.20 ± 0.10; see Table II). Interestingly, the increase in the immunoprecipitation by anti-α1 was observed in three of the four aged hippocampal samples tested, emphasizing the heterogeneity of the aging process at the hippocampal formation (the individual data were 57.8, 71.4, 66.4, and 65.0% for the aged samples).

These results demonstrated that the age-dependent increase in the γ2 subunit, mRNA and protein, was not reflected by a similar increase of the γ2 incorporated in the mature $GABA_A$ receptor. Thus, the amount of γ2 subunit, lacking benzodiazepine binding activity, should increase in the aged hippocampus. We tested this possibility by quantifying the volume (in μl) of anti-γ2 antiserum needed to immunoprecipitate a fixed (and identical in both ages) amount of [^{3}H]flumazenil binding activity. As expected, Fig. 6, the immunoprecipitation curves were clearly shifted to the right with no changes in the maximal immunoprecipitation (see also Ref. 23) (64.1 ± 3.6%, n = 3, and 58.5 ± 2.1%, n = 3, for adult and aged hippocampi, respectively). The curves were fitted to a monoexponential decay function, and the volume of antibody that produces a half-maximal immunoprecipitation was determined. The volume of anti-γ2 for half-maximal immunoprecipitation was 3.3 ± 0.7 μl, n = 3, and 8.5 ± 0.7 μl, n = 3, for adult and aged hippocampus, respectively (significant difference between both ages; ANOVA F (1,4) = 82.64, p = 0.0008; Tukey $p < 0.01$). These results demonstrated the existence of a significant increase in the amount of γ2 subunit that exhibit no binding activity (probably unassembled subunit). This increase (aged/adult ratio, 2.60 ± 0.21) is similar to that observed for γ2S version at the mRNA level (3.53 ± 0.54) and the γ2 protein (2.90 ± 0.62).

Expression of the AMPA Receptor Subunits—The relative proportion of the different subunits of the AMPA receptor (GluR1–4) was studied using the cDNA from the same adult and aged hippocampal preparations described in Fig. 2. As shown in Fig. 7*A,* the relative proportion of the different AMPA receptor subunits, from both adult and aged hippocampus, agree with that previously reported (33). As also shown, no gross changes with aging were detected. Similarly to the

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TABLE II

Total benzcdiazepine binding activity and anti-γ2 or anti-α1 immunoprecipitation in adult and aged hippocampus

The isolated hippocampal membranes were solubilized, and the total binding activity of 10 nM [^{3}H]flumazenil were determined. For immunoprecipitation experiments, 150 μg of solubilized proteins were immunoprecipitated by two sequential incubations with 50 μl + 50 μl of anti-γ2 or 10 μl + 10 μl of anti-α1. The binding activity (10 nM [^{3}H]flumazenil) was determined in both pellets and in the final supernatant. The results (mean ± S.D.) represent the binding activity of the solubilized receptor (total binding) or the cumulative immunoprecipitation and are expressed in pmol/mg solubilized protein or percentage of immunoprecipitation.

Age	n	Solubilized receptor total binding	[^{3}H]Flumazenil binding activity: cumulative Immunoprecipitation			
			Anti-γ2		Anti-α1	
		pmol/mg protein	*pmol/mg protein*	*%*	*pmol/mg protein*	*%*
3 months	4	1.51 ± 0.14	1.36 ± 0.07	90.9 ± 4.60	0.81 ± 0.06	54.5 ± 3.8
24 months	4	1.15 ± 0.08[a]	1.05 ± 0.08[b]	91.5 ± 3.03	0.74 ± 0.07	65.1 ± 5.6[c]

[a] Significant decrease from adult ANOVA F(1,6) = 15.13, p = 0.008; Tukey $p < 0.05$.
[b] Significant decrease from adult ANOVA F(1,6) = 33.47, p = 0.0001; Tukey $p < 0.01$.
[c] Significant increase from adult ANOVA F(1,6) = 9.75, p = 0.02; Tukey $p < 0.05$.

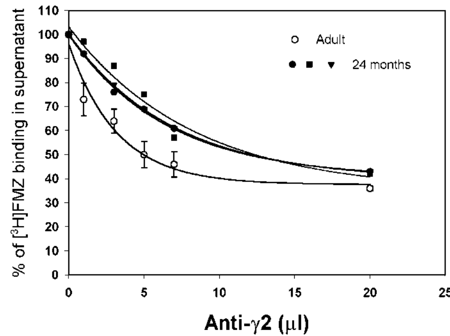

FIG. 6. **Immunoprecipitation curves of the anti-γ2 antibody in adult and aged hippocampus.** The adult and aged hippocampal membranes were solubilized, and a fixed amount of [^{3}H]flumazenil (10 nM) binding activity (7, 500 ± 500 cpm), equal for both ages, was immunoprecipitated with increasing volumes of anti-γ2 antiserum. After immunprecipitation, the supernatants were collected and the binding activity was determined. The results, expressed as a percentage of the binding activity in absence of antibody, are shown as mean ± SD of three different experiments in adults or individually for the aged rats. All experiments were performed in duplicate.

GABA$_A$ receptor, the coefficient of variation increased with aging (0.05, 0.04, 0.04, and 0.5 for GluR1–4 in adults, respectively (mean, 0.15), and 0.18, 0.05, 0.12, 0.66 for the same subunits in the aged rats (mean, 0.25)) indicating the heterogeneity of the aging process. Considering all subunits and both age groups, Fig. 7*A,* significant differences were observed (Multifactor ANOVA Subunit × Ages F (3, 41) = 7.68, p = 0.004). The proportion of GluR1 slightly decreased in the aged samples (ANOVA F (1,10) = 8.87, p = 0.016; Tukey $p < 0.05$), whereas the GluR3 showed a tendency to increase (ANOVA F (1,10) = 7.6, p = 0.02; Tukey $p < 0.05$). When the modifications (*versus* adult) were considered individually (Fig. 7*B*), a predominant pattern was observed. In all six animals, GluR1 decreased (ranging from –9 to –38%; mean, –18.4 ± 13.4%), GluR3 increased (ranging from 20 to 65%; mean, 39.8 ± 15.3%), and GluR2 showed no variations (mean, –2.6 ± 4.5%). However, despite these age-dependent modifications, we found no significant differences between either ages in the (GluR1 + GluR3)/GluR2 ratio (GluR4 is a minor component of the AMPA receptor): 1.57 ± 0.17, n = 4, and 1.55 ± 0.17, n = 6, for adult and aged hippocampus, respectively (Fig. 7). Thus, the calcium permeability of the AMPA receptors seems not to be altered during aging.

We also quantified the relative proportion between the flip/flop splicing version of each subunit. No differences were observed (not shown).

Finally, the total expression of the AMPA receptor (GluR1 + GluR2 + GluR3 + GluR4 subunits) were determined in relation to the expression of the β-actin. As shown in Fig. 8, no age-dependent modifications were detected (AMPA/β-actin ratio: 0.31 ± 0.04, n = 4, and 0.34 ± 0.05, n = 6, for adult and aged hippocampus, respectively).

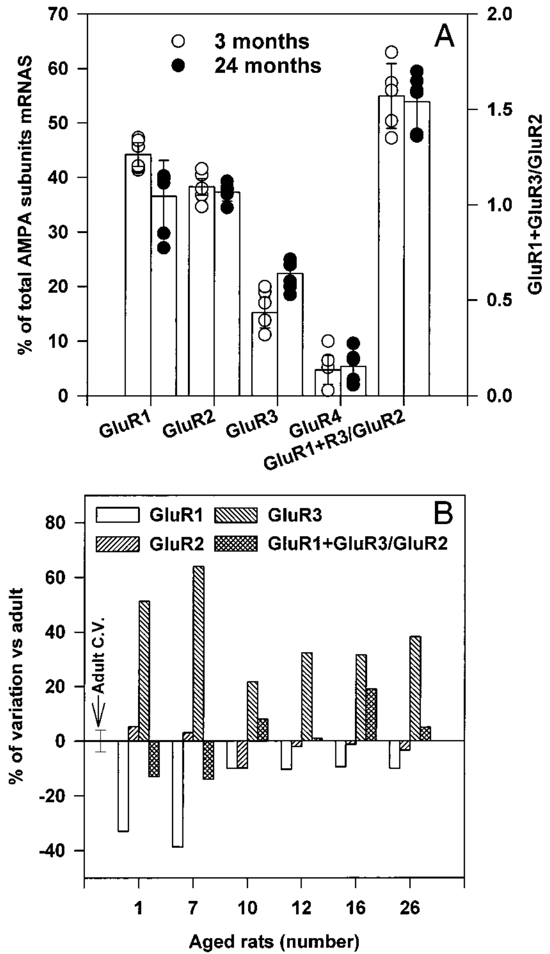

FIG. 7. **Expression of the AMPA subunits in adult and aged rat hippocampus.** *A,* shown is the proportion of the GluR1–4 AMPA receptor subunits expressed in the adult (*open circles*) and aged (*closed circles*) rat hippocampus. The results are shown individually and as mean ± SD (*bars*) of at least five adult and six aged rats. *B,* the percentage of variation (in relation to the adult values) of GluR1, GluR2, and GluR3 subunits was determined individually for each analyzed rat. The coefficient of variation of these subunits in the adult samples was also indicated in the figure.

Expression of the GAD65/67 Isoenzymes—The expression of both isoforms of the GAD enzyme (GAD65 and GAD67) was also analyzed using the same experimental approach as for the AMPA receptor in the same adult and aged samples. The results (Fig. 8) indicate the absence of significant differences between both ages.

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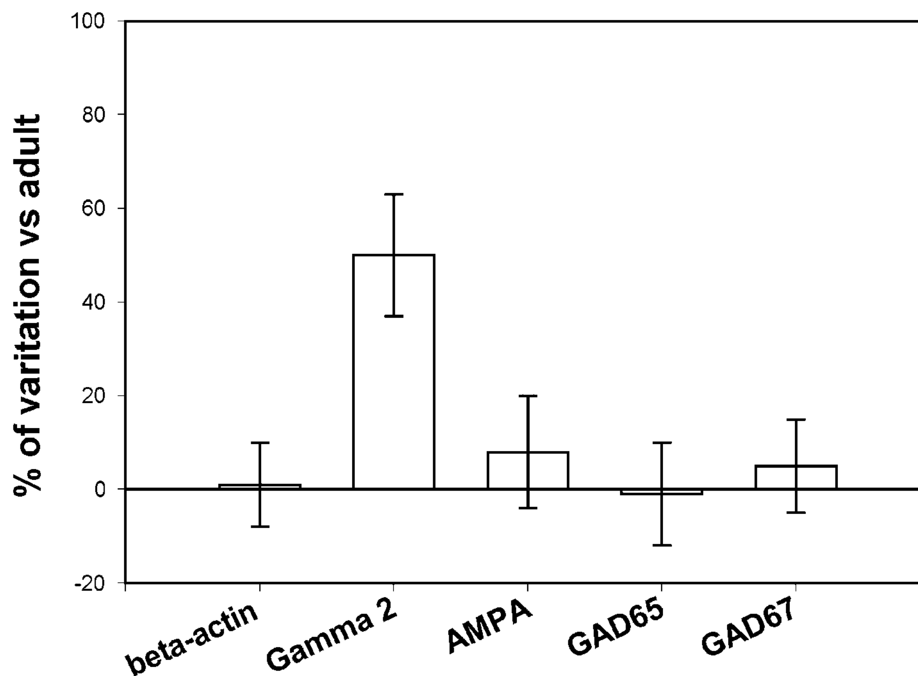

FIG. 8. **Absence of variation on the expression of the total AMPA receptor subunits and both GAD isoenzymes in the aged hippocampus.** The expression of total AMPA receptor subunits (GluR1 + GluR2 + GluR3 + GluR4) and both GAD isoenzymes (GAD65 and GAD67) were determined in relation on the expression of the β-actin. The results (mean ± SD) from four adult and six aged samples are expressed as the percentage of variation *versus* adult. For the β-actin, the PCR products from both ages were directly compared. Also, the age-dependent variation on the γ2S subunit of the GABA$_A$ receptor was determined, in parallel, as internal control of the method.

DISCUSSION

In this study, we have determined the expression of eight subunits of the GABA$_A$ receptor and all four subunit of the AMPA receptor in hippocampus from adult and six aged rats.

Age-dependent Modifications in the Expression of the GABA$_A$ and AMPA Receptors—The main observation of this work is the existence of an age-related increase on the expression of the γ2S subunit and, to a minor degree, in the α1 subunit of the hippocampal GABA$_A$ receptor, in absence of major modification of the AMPA receptor, in the same aged hippocampi.

The mRNA (short version) and protein of the γ2 subunit increased dramatically in all aged hippocampal samples. Thus, in our rat population, the increase in the expression of the γ2 subunit could be considered a hallmark of the aging process of the hippocampal GABA$_A$ receptor. On the other hand, the expression of the different α subunits also increases with aging; however, this increase is lower in magnitude and also varies between the different aged animals. In most cases, two different α subunits increased; an increase in α1 subunit (mRNA and protein) was the most common pattern. The reason for this heterogeneous profile is not clear, but this profile likely means that critical variables affecting the GABA$_A$ subunit expression are different in the different aged rats (see below).

The age-dependent modifications in the pharmacological (8, 9) and the electrophysiological (11) properties of the GABA$_A$ receptor are analogous to those found in the substantia nigra in response to degeneration of the GABAergic striatal afferents (34, 35). Indeed, the observed age-dependent increase in the expression of the different GABA$_A$ receptor subunits in the hippocampus could represent a normal response of the neurons to a deafferentiation process. Interestingly, Shetty and Turner (15) have demonstrated a decrease in the number of the GABAergic neurons at the hippocampal formation of the aged rats. Thus, a decline in the number of the interneurons could indicate a reduction of the GABAergic inputs to the principal cells and, consequently, an increase of the expression of the GABA$_A$ receptor complex. However, in our adult and aged hippocampi, no apparent differences in the expression of both GAD isoenzymes were observed. This discrepancy does not invalidate our hypothesis because: (i) a deafferentiation process, without net neuronal loss, would result in an increase of the expression of the GABA$_A$ receptor, or (ii) it is possible that the surviving interneurons increase the expression of these isoenzymes compensating for the loss in the neuronal number (1). Thus, we propose that the age-dependent increase of the expression of the GABA$_A$ receptor subunits constitutes a normal adaptive response of the principal cells to deficiencies on the GABAergic system.

On the other hand, the age-dependent reduction in the GABAergic cell number also was heterogeneous (15). Therefore, it is tempting to speculate that the high interanimal heterogeneity found in the expression of the different α subunits may reflect the reduction of different GABAergic populations and/or deafferentiation of a particular subset of synaptic contact in the principal cells of the aged hippocampus. In consequence, depending on the different GABAergic synapses affected, the adaptive response may differ in the different animals. It is important to emphasize the dramatic increase in the expression of the γ2 subunit (mRNA and protein) in all tested aged hippocampi, indicating the existence of a similar process in the whole aged population.

Our results also demonstrated the absence of major modifications on the expression of the hippocampal AMPA receptor subunits in the same animals. The absence of such modifications is consistent with previous reports demonstrating the absence of changes of the [^{3}H]AMPA binding sites at the hippocampal formation (12, 13, 36) and suggest the absence of major modifications on the excitatory inputs. This proposal is based on the fact that the expression of the AMPA receptor subunits, GluR1 and GluR2/3, also increased after deafferentiation (37). This conclusion is also in accordance with the preservation of the principal neurons observed in the aged animals (38).

We also observed a decrease in the relative proportion of the GluR1 subunit concomitant with an increase in the GluR3 subunit. These modifications might reflect an increase on the excitability of the aged hippocampus, because similar alterations (decrease in GluR1 and increase in GluR3) have been reported in response to lesion-induced limbic seizures (39) or in a pilocarpine model of spontaneous limbic epilepsy (40).

As a whole, the observed age-dependent modifications in the expression of both GABA$_A$ and AMPA receptor subunits

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strongly suggest a preferential age-dependent alteration of the GABAergic cells that could suggest a decrease in the inhibitory system (see also Refs. 1, 41, and 42).

Age-dependent Modifications of the Mature $GABA_A$ Receptor—The increase in the expression of both $\alpha 1$ and $\gamma 2$ subunit, mRNA, and protein could have a direct repercussion on the mature $GABA_A$ receptor in membranes. Thus, we investigated the anti-$\alpha 1$ and anti-$\gamma 2$ immunoprecipitation of the [^{3}H]flumazenil binding activity, solubilized from isolated membranes. We presume that only mature (assembled) $GABA_A$ receptors display benzodiazepine binding sites (43, 44). In fact, our immunoprecipitation experiments demonstrated that the increased expression of the $\alpha 1$ subunit was directly reflected by an increase in the proportion of $GABA_A$ receptor containing this subunit in the aged hippocampus, confirming previous pharmacological and immunological experiments (8–10). Therefore, the increased transcripted and translated $\alpha 1$ subunit is incorporated into the assembled receptor, modifying the pharmacological and electrophysiological properties of the $GABA_A$ receptor (8, 9, 11).

On the other hand, the increased expression of the $\gamma 2$ subunit is not reflected by modifications in the mature $GABA_A$ receptor complex. The reduction of both the total and the anti-$\gamma 2$ immunoprecipitated [^{3}H]flumazenil binding activity could indicate the existence of an increase in the unassembled, or partially assembled, $\gamma 2$ subunits (displaying no benzodiazepine binding activity) as confirmed by the increase in the anti-$\gamma 2$ antibody volume that produced a half-maximal immunoprecipitation in the aged hippocampus. Therefore, the increase on the expression of the $\gamma 2$ subunit could result in an accumulation of this subunit in, probably, intracellular compartments. We do not know the reasons for this differential effect of aging on the incorporation of the $\alpha 1$ and $\gamma 2$ subunits to the mature receptors, but the extremely high increase on the expression of the $\gamma 2$ subunit, more than 12 times higher than that of the α subunits (see Table I) in the absence of apparent modifications in the expression of the β subunits, could exceed the capacity of the assembling process of the $GABA_A$ receptor.

In conclusion, our results demonstrated the existence of an age-dependent increase on the expression of the $GABA_A$ receptor subunits in the hippocampal formation with minor modifications on the expression of the AMPA receptor. These modifications indicate the existence of an specific alteration (neuronal loss and/or deafferentiation) in the GABAergic system.

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$GABA_A$ and α-Amino-3-hydroxy-5-methylsoxazole-4-propionate Receptors Are Differentially Affected by Aging in the Rat Hippocampus

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