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GABA$_B$ Binding Sites in Early Adult and Aging Rat Brain

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TURGEON, S. M. AND R. L ALBIN. *GABA$_B$ binding sites in early adult and aging rat brain.* NEUROBIOL AGING **15**(6) 705–711, 1994.—The effect of aging on GABA$_B$ binding was investigated in rat brain. Receptor autoradiography was used to investigate both GABA$_B$ and GABA$_A$ binding at 2 months, 3 months, 13 months, and 23 months. GABA$_B$ binding decreases significantly between 2 months and 23 months of age, as does GABA$_A$ binding, with was investigated in rat brain. Receptor autoradiography was used to investigate both GABA$_B$ and GABA$_A$ binding at 2 months, 3 months, 13 months, and 23 months. GABA$_B$ binding decreases significantly between 2 months and 23 months of age, as does GABA$_A$ binding, with the greatest decrease between 2 and 3 months. The decrease in GABA$_B$ binding appears to be due to a decrease in binding site affinity rather than a decrease in receptor density. The noncompetitive GABA$_B$ antagonist zinc, the competitive GABA$_B$ antagonist CGP 35348, and the guanyl nucleotide analogue GTP-γ-S all inhibit GABA$_B$ binding identically in 2 month and 23 month brain. These data indicate subtle age-related changes in the GABA$_B$ binding in early adult life but little change with senescence.

GABA$_B$ GABA$_A$ Aging Zinc GTP-γ CGP 35348

γ-AMINOBUTYRIC acid (GABA) is the predominant inhibitory neurotransmitter in the central nervous system. A number of GABAergic parameters have been reported to undergo changes during aging. Cerebrospinal fluid GABA levels decrease with age in humans (19,37) and rat brain GABA levels have been reported either to remain stable (10) or decrease with age (2). Contradictory studies have reported both increased (36) and decreased (43) high-affinity GABA uptake in aged rat brain. Both GABA release (1) and glutamic acid decarboxylase (GAD) activity (34) appear to decrease with age.

The GABA$_B$ receptor is one of two known central nervous system receptors for GABA. The other receptor, the GABA$_A$ receptor, is a ligand gated ion channel. GABA$_B$ receptors are G-protein linked (15,22) and activated by the GABA$_B$ selective agonist baclofen (4,5). Receptor activation can lead to either inhibition of Ca^{2+} currents (15,16,25) or activation of K^+ currents (17,32) and can act through second messenger systems to modulate cyclic AMP formation (21,42,44).

Most investigations of age-related changes in GABA receptors have focused on the GABA$_A$ receptor. Several studies report the absence of age-related changes in GABA$_A$ receptor binding (26, 27,30,41). However, one study found selective decreases in binding in the substantia nigra and hypothalamus with age (18). In addition, decreased ^{35}S-TBPS binding to the GABA$_A$ receptor associated picrotoxin binding site was observed in aged animals (11). Whereas age-related increases in [^{3}H]diazepam binding to benzodiazepine binding sites and increased enhancement of GABA$_A$ binding by benzodiazepines have been reported (9), the sensitivity of benzodiazepine binding to modulation by GABA$_A$ receptor binding does not appear to be altered in aged animals (24). Finally, functional studies have shown increased GABA inhibition of firing in hippocampal pyramidal cells (26) and decreased GABA$_A$ receptor-stimulated Cl- uptake in membrane vesicles (11) in aged rat brain.

In contrast, the literature on age-related changes of the GABA$_B$ receptor is quite sparse. We have previously reported that GABA$_B$ binding site distribution undergoes several regional changes during early postnatal development which can be correlated with changes synaptogenic and organizational events (39). However, the only study investigating GABA$_B$ receptors in aging has reported that postsynaptic GABA$_B$ responses in hippocampal pyramidal cells decrease in aged rats (33). In light of evidence that GABA$_B$ receptors are implicated in memory processes (7) and that GABA$_B$ receptor binding is altered in Alzheimer's disease (AD) (12,13), we believe that further investigation of changes in GABA$_B$ receptor binding in aging is necessary. We have used [^{3}H]GABA receptor autoradiography to describe the regional changes in GABA$_B$ binding and GABA$_B$ binding site pharmacology in rat brain across a wide spectrum of ages. Regional binding results are compared with parallel studies of GABA$_A$ binding.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]GABA (91.7 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Isoguvacine was purchased from Cambridge Research Chemicals (Cambridge, England). (±)Baclofen was a gift from Dr. R. Lovell (Ciba-Geigy, Summit, NJ). CGP 35348 was donated by Drs. H. Schroter and L. Maitre (Ciba-Geigy, Basel, Switzerland). GTP-γ-S was purchased from Sigma (St. Louis, MO).

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Animals

Male Fisher 344/BNNia rats were obtained from Charles River (New York, NY) through the National Institute of Aging. For distribution studies, brains were used from animals aged 2 months (n = 6), 3 months (n = 4), 13 months (n = 6), and 23 months (n = 6). For the saturation and competition assays, brains were used from 2 month (n = 6; saturation, n = 3; competition) and 23 month old animals (n = 6; saturation, n = 3; competition).

Tissue Preparation

For autoradiography experiments, animals were decapitated and their brains were rapidly dissected and frozen in Lipshaw embedding matrix surrounded by powdered dry ice. Brains were mounted on cryotome chucks with Lipshaw embedding matrix. Serial sections (20 μm, in the horizontal plane) were cut on a Lipshaw cryostat at −20°C and thaw mounted onto gelatin-coated slides. Sections were stored for no longer than 24 h at −20°C prior to assays.

[^{3}H]GABA Quantitative Autoradiography

$GABA_B$ binding sites were examined with 20 nM [^{3}H]GABA in the presence of 10 μM isoguvacine while $GABA_A$ binding sites were examined with 20 nM [^{3}H]GABA in the presence of 100 μM baclofen (14). Sections were run in duplicate. Sections were prewashed for 30 min in buffer containing 50 mM Tris-HCl and 2.5 mM $CaCl_2$ (pH 7.4 at +4°C) to remove endogenous ligand and were then dried under a stream of cool air. Assay conditions involved a 45-min incubation at +4°C with 20 nM [^{3}H]GABA in 50 mM Tris-HCl and 2.5 mM $CaCl_2$ (pH 7.4 at +4°C). In the pharmacology experiments, concentrations of 30 nM to 1 mM CGP 35348, 10 nM to 100 μM GTP-γ-S, and 1 nM to 3 mM zinc were included in the incubation mixture. Saturation experiments were

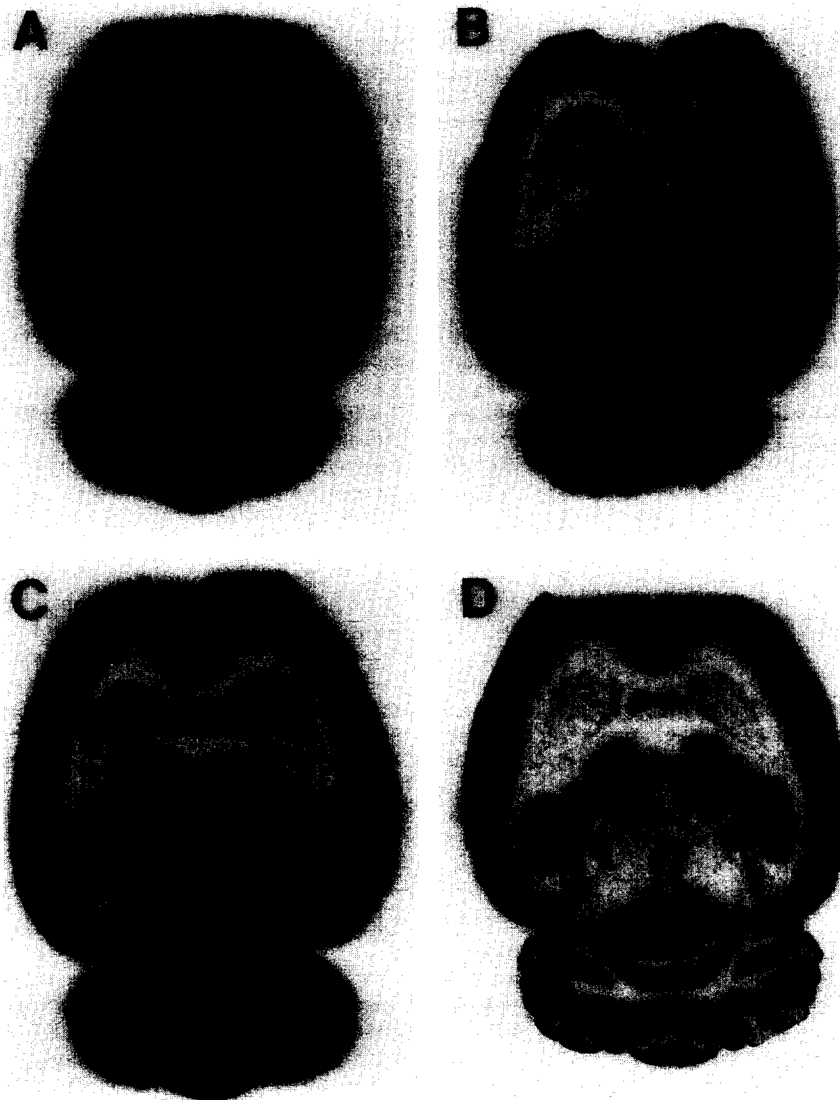

FIG. 1. Autoradiographs of [^{3}H]GABA binding to $GABA_B$ binding sites in 2- (A), 3- (B), 13- (C), and 23-month-old brain (D). Representative sections for each age group were chosen from animals whose individual $GABA_B$ binding levels most closely approximated the overall average binding levels for that age group.

performed by the method of isotopic dilution with total GABA concentrations from 666 pM to 33.3 μM. For concentration points at and below 33.3 nM, only [^{3}H]GABA was used. For concentration points above 33.3 nM, 33.3 nM [^{3}H]GABA was diluted with nonradioactive GABA. Nonspecific binding was determined by the addition of 100 μM (+)baclofen. Following incubation, slides were removed individually and rinsed quickly 3 times with 3 ml buffer squirted from a repipetter and once with 3 ml 2.5% glutaraldehyde in acetone and immediately blown dry with warm air. Slides were mounted in an X-ray cassette and apposed to tritium sensitive film (^{3}H-Hyperfilm, Amersham) along with standards containing known amounts of radioactivity (ARC, Inc., St. Louis, MO) for 3–4 weeks at +4°C. Films were developed for 4 min in Kodak D19, fixed and dried.

Data Analysis

Ligand binding was quantified with computer-assisted densitometry using an MCID system (Imaging Research Inc., St. Catherine's, Ontario). To quantify ligand binding levels, the optical density of co-exposed standards was determined and a standard curve was generated by fitting standard optical density values to standard radioactivity values with a fourth-degree polynomial regression equation as described previously (31). Standards were commercial ^{14}C plastic standards (ARC Inc., St. Louis, MO), calibrated against previously described ^{3}H-brain paste standards constructed to give a known amount of radioactivity per mg protein (31). Optical density readings were taken in regions of interest and converted to fmoles/mg protein values using the standard curve derived from the optical density values of the standards (31).

One-way ANOVAs were performed to assess age-related variance in regional $GABA_B$ and $GABA_A$ binding levels and posthoc comparisons between individual groups were made with Scheffeé F tests. IC_{50} values were calculated in 4 regions for zinc and CGP 35348 and in the cerebellar molecular layer for GTP-γ-S with a dose–response semi-log plot. Unpaired student t tests were used to compare IC_{50} values in young and aged rats. Hill numbers for CGP 35348 were determined by generating Hill plots as described by Bylund (8) and analyzed for statistical significance with a one group t test. CGP 35348 inhibition curves and saturation data were analyzed using the computer program LIGAND (Biosoft, Furguson, MO). LIGAND performed a statistical analysis of the residual variance of one- versus two-site curve fits for the combined data from 6 animals from each age group at each saturation point using a partial F test. A two-site fit was considered to be preferable when the residual variance for a two-site fit was significantly lower than the residual variance for a one-site fit at significance levels of $p < 0.05$.

RESULTS

Regional Distribution of $GABA_B$ and $GABA_A$ Binding in Aging

$GABA_B$ binding decreased significantly from two months to 23 months in all regions examined while significant decreases in $GABA_A$ binding were seen in cerebellar granule cell layer, thalamus, striatum, and the CA1 and CA3 regions of the hippocampus (Fig. 1 and Fig. 2) and in the cerebellar granule cell layer (GCL; data not shown). Due to the high level of binding in the GCL, the data is not presented on this graph. Posthoc comparisons of indi-

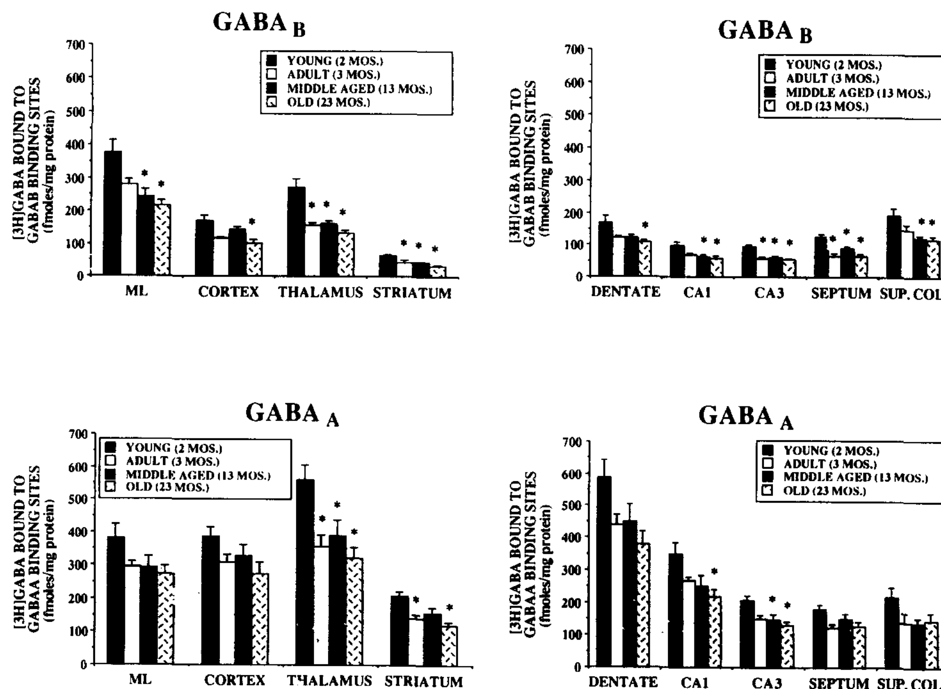

FIG. 2. [^{3}H]GABA binding to $GABA_B$ and $GABA_A$ binding sites in 2- (n = 6), 3- (n = 4), 13- (n = 6), and 23-month-old (n = 6) rats. Analysis of variance (ANOVA) revealed significant changes in $GABA_B$ binding across age groups in all regions ($p < 0.05$, one-factor ANOVA) and significant changes in $GABA_A$ binding across age groups in the thalamus, striatum, dentate, CA1, CA3, and septum ($p < 0.05$, one-factor ANOVA). Posthoc tests revealed significant decreases in $GABA_B$ binding between the 2-month-old group and some older groups in all regions and significant decreases in $GABA_A$ binding between the 2-month-old group and some older groups in the thalamus, striatum, CA1, and CA3. Error bars represent SEM values. *($p < 0.05$, as compared to the 2-month-old group, Scheffeé F test).

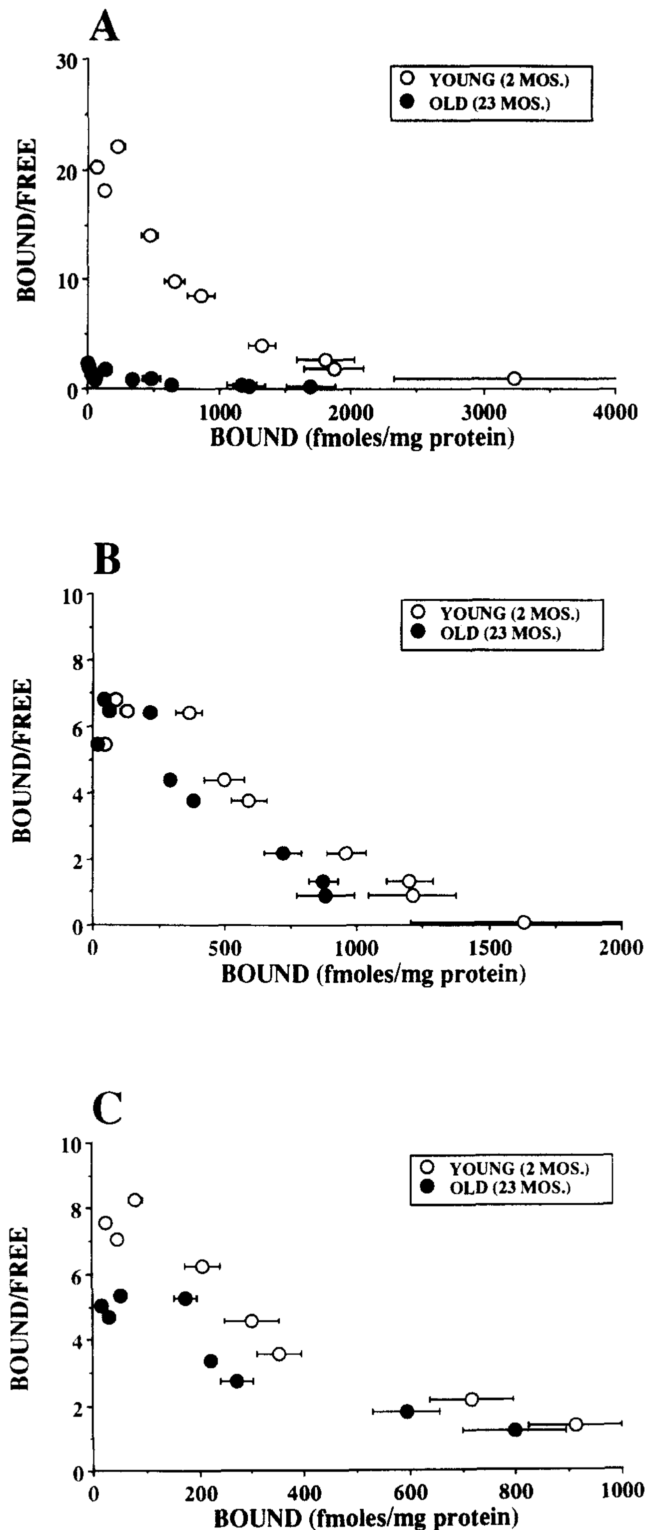

FIG. 3. Scatchard analysis of [^{3}H]GABA binding to $GABA_B$ binding sites in the cerebellar molecular layer (A), thalamus (B), and cortex (C) of 2- (○) and 23-month-old (●) rats. The plot is nonlinear, indicating the presence of more than one binding site. A two-site fit for [^{3}H]GABA binding to $GABA_B$ binding sites was preferred in all regions at both ages as determined by a nonlinear regression of the saturation data and a partial F test using the LIGAND program ($p < 0.005$). Each point represents average binding levels from six animals. Error bars represent SEM values.

vidual age groups revealed that the only significant changes in binding were those observed between the 2-month group and the older groups. In the GCL, $GABA_A$ binding in all older age groups was significantly less than in the 2-month group. No significant decreases in binding were observed between individual age groups after 3 months of age.

Saturation Analysis

Analysis of the saturation data using the computer program LIGAND suggests that [^{3}H]GABA binds to two $GABA_B$ binding sites in the cerebellar molecular layer, thalamus, and neocortex in both 2-month and 23-month animals. A two-site fit was preferred in all of these regions with significance levels of $p = 0.002$ or lower. Scatchard curves generated from saturation data in both 2- and 23-month-old animals also illustrate a two-site fit for [^{3}H]GABA binding to GABAB binding sites in the cerebellar molecular layer (Fig. 3). Both K_D and B_{max} values for the high and low affinity sites increased in aged animals in all regions except the thalamus were the B_{max} was not increased for the high affinity site (Table 1).

Competition Assays

$GABA_B$ binding was displaced equipotently in young and aged brains by the noncompetitive $GABA_B$ antagonist zinc and the competitive $GABA_B$ antagonist CGP 35348 (Fig. 4 A and B). The IC_{50} values for these compounds are reported in Table 2.

Whereas inhibiting $GABA_B$ binding at higher doses, zinc also enhanced $GABA_B$ binding at lower doses. In the cerebellar molecular layer, $GABA_B$ binding was enhanced to 136.0 % ± 10.7% by 30 mM zinc and to 127.9% ± 8.5% ($p < 0.05$, one-tailed t test) by 100 mM zinc in 2-month-old brains, and to 133.1 % ± 17.8% by 30 mM zinc and to 118.5% ± 7.7% by 100 mM zinc in 23-month-old brains. Similar levels of enhancement were observed in the dentate and neocortex but not in the thalamus.

Hill plots and equilibrium binding analysis were generated for the inhibition of $GABA_B$ binding by CGP 35348 in the cerebellar molecular layer. The Hill coefficient for the young animals was significantly different than 1 (nH = 1.313 ± 0.016, $p < 0.05$, two-tailed t test) while in adult animals a nonsignificant trend away from 1 was observed (n_H= 1.502 ± 0.190). Equilibrium binding analysis using the LIGAND computer program revealed that, in young animals, CGP 35348 displaces binding from both a high affinity site (K_I = 5.0 μM, B_{max} = 22.8 fM) and a low affinity site (K_I = 78.5 μM, B_{max} = 3.2 fM). However, in aged animals a single site fit was preferred (K_I = 17.8 μM, B_{max} = 6.6 fM).

TABLE 1

K_D AND B_{MAX} VALUES FOR $GABA_B$ BINDING IN YOUNG ADULT AND AGED RATS

	ML	Cortex	Thalamus
Young (2 months)			
K_D high (nM)	23.9	30.3	41.7
B_{max} (fM/mg protein)	20.9	7.7	21.0
K_D low (nM)	700	377	1150
B_{max} (fM/mg protein)	80.4	31.0	47.0
Aged (23 months)			
K_D high (nM)	70.7	54.9	54.4
B_{max} (fM/mg protein)	36.0	10.6	17.0
K_D low (nM)	4800	1610	1510
B_{max} (fM/mg protein)	168.0	47.0	57.0

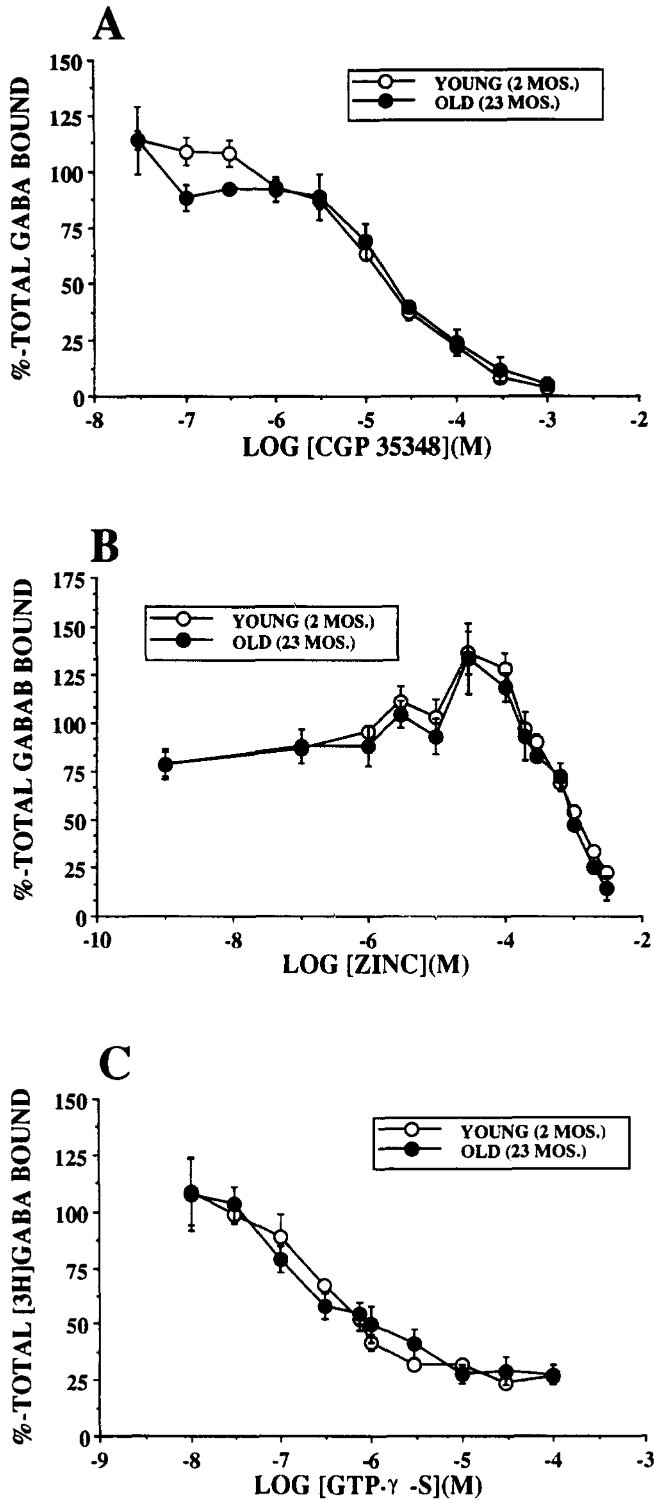

FIG. 4. Inhibition of [^{3}H]GABA binding to the GABA$_B$ binding site in the cerebellar molecular layer of 2- (○) and 23-month-old (●) old rats by the competitive GABA$_B$ antagonist CGP 35348 (A), the noncompetitive GABAB antagonist zinc (B), and the guanyl nucleotide analogue GTP-γ-S (C). Each point represents average binding levels from three animals. Error bars represent SEM values. No significant change in IC$_{50}$ values from 2- to 2-months was observed for any of these compounds. CGP 35348 was found to inhibit [^{3}H]GABA binding to two GABA$_B$ binding sites with different affinities for the antagonist (see Results section).

In addition, the guanyl nucleotide analogue GTP-γ-S displaced GABA$_B$ binding equipotently in both young and aged animals (Fig. 4C) with IC$_{50}$s in the cerebellar molecular layer of 763.4 + 108.1 nM and 619.1 + 237.1 nM, respectively.

DISCUSSION

Regional Distribution of GABA$_B$ Binding in Aging

Our results indicate an age-related decrease in GABA$_B$ binding in all regions examined without qualitative changes in the regional distribution of binding. GABA$_B$ binding also exhibited a downward trend in most regions with aging, however, significant decreases in binding were found only between the 2-month-old group and the older groups. Taken in combination with our previous data (39), these results indicate continuous decreases in GABA$_B$ binding from the time where GABA$_B$ binding peaks during the first 2–3 postnatal weeks of life until the third postnatal month, from which point GABA$_B$ binding remains fairly stable throughout adult life.

The drop in GABA$_B$ binding between 2 and 3 months may coincide with other changes observed around puberty. GABA$_B$ binding has been reported to be more sensitive to pertussis toxin at 10–12 weeks of age than at earlier ages, indicating changes in receptor-G-protein linkage (23). Whereas the authors of this report did not comment on changes in overall binding levels, their figures also indicate decreases in GABA$_B$ binding between 7- to 8-week-old animals and 10- to 12-week-old animals in some regions (23).

GABA$_A$ binding was also found to decrease in some regions. Our results are not necessarily in disagreement with previous studies showing the absence of significant changes in GABA$_A$ binding with age as most of these studies used 3-month-old or older rats as their young age group. Whereas we did find a downward trend in GABA$_A$ binding with age, the only significant age-related decreases were found in comparison to the 2-month-old group.

Saturation Analysis

Saturation studies were performed to assess the nature of the decrease in GABA$_B$ binding observed between 2 months and 23 months of age. [^{3}H]GABA appears to be binding to both a high and a low affinity GABA$_B$ binding site at both ages in the cerebellar molecular layer, neocortex, and thalamus, as a two-site fit was preferred by the computer program LIGAND for GABA$_B$ binding in these regions. This finding is in agreement with our previous observation of a two-site fit for GABA$_B$ binding in early postnatal and young adult Sprague–Dawley rat brain (39).

The decrease in binding seen between 2 months and 23 months appears to be due to changes in the affinity of the receptors rather than a decrease in receptor number. Both K_D and B_{max} values for high and low affinity binding sites increased between 2 months and 23 months. The observation of decreased affinity and increased receptor number in the presence of decreased binding suggests that the reduction in binding is due to a decrease in the affinity of the binding site for the ligand, rather than a decrease in receptor number. Furthermore, an analysis of the predicted change in binding using the K_D and B_{max} values in the equation $B = ((B_{max}1[F])/(K_D1 + [F])) + ((B_{max}2[F])/(K_D2 + [F]))$ predicts a 43% decrease in GABA$_B$ binding in the cerebellar molecular layer whereas the actual decrease in binding observed is 42%, supporting the accuracy of the observed changes. Changes in GABA$_B$ binding site affinity between 2 months and 23 months are not surprising in light of the changes in GABA$_B$ receptor G-protein linkage reported following puberty (23).

The observed increase in binding site affinity in aged animals suggests that a different type of GABA$_B$ receptor is present in

TABLE 2

REGIONAL IC_{50} VALUES FOR INHIBITION OF $GABA_B$ BINDING BY ZINC AND CGP 35348

	ML	Cortex	Thalamus	Dentate
Zinc (mM)				
2 mos.	1.14 ± 0.94	1.073 ± 0.24	0.75 ± 0.23	1.37 ± 0.39
23 mos.	0.94 ± 0.03	1.206 ± 0.22	0.80 ± 0.08	1.19 ± 0.21
CGP 35348 (μM)				
2 mos.	18.04 ± 2.04	47.13 + 9.35	58.75 + 16.87	22.77 + 9.80
23 mos.	19.63 ± 3.09	31.12 + 13.01	33.35 + 8.82	15.06 + 4.51

Values are mean ± SEM.

these animals than in the young adults. This difference may result from a change in the G-protein coupling ability of the receptor. However, a change in the proportion of receptor subtypes with different affinities for GABA could produce the same result. In either case, the presence of $GABA_B$ receptor heterogeneity is suggested by these data.

Competition Assays

The noncompetitive $GABA_B$ antagonist zinc, the competitive antagonist CGP 35348, and the guanyl nucleotide analogue GTP-γ-S all displace $GABA_B$ binding identically in 2-month-old brain and 23-month-old brain.

Age-related changes in the effect of zinc on $GABA_B$ binding have been observed during development. Zinc displaces $GABA_B$ binding more potently during early postnatal development as compared to adulthood (39). In addition, zinc enhances $GABA_B$ binding in certain regions at lower doses in adult brain (40) but not early postnatal brain (39). Whereas the enhancement of $GABA_B$ binding by low concentrations of zinc is only significant in the molecular layer of 2-month-old animals, the absence of significant changes elsewhere is probably due to the relatively large SE and small *n*. Because the binding curves for 2- and 23-month-old brains appear virtually superimposable and significant enhancement has been observed previously (40), we suggest that zinc is enhancing $GABA_B$ binding similarly in both age groups.

The competitive $GABA_B$ antagonist CGP 35348 also displaced $GABA_B$ binding identically in 2- and 23-month-old rat brain. Computer analysis of this inhibition using LIGAND demonstrated that CGP 35348 is displacing $GABA_B$ binding from both a high and a low affinity binding site in the cerebellar molecular layer of 2-month-old brain but from only one site in the 23-month-old brain. Decreased overall levels of $GABA_B$ binding in the older animals lead to increased variability which may be preventing the curve fitting program from obtaining a preferable two-site fit in these brains. Displacement curves for CGP 35348 appear to be superimposable, suggesting that inhibition of $GABA_B$ binding by CGP 35348 is the same in both young and aged animals.

The observation of Hill numbers significantly greater than one for the displacement of $GABA_B$ binding by CGP 35348 in the cerebellar molecular layer of 2-month-old brains also suggests a complex interaction of this compound with $GABA_B$ binding sites. Because antagonists cannot discriminate between high and low affinity receptor states, the observation of a two-site fit for CGP 35348 inhibition suggests that there are two different populations of GABAB receptors with different affinities for CGP 35348. Our results present further evidence for multiple subtypes of $GABA_B$ receptors in rat brain (3,6,35).

Finally, the guanyl nucleotide analogue GTP-γ-S inhibits $GABA_B$ binding in 2- and 23-month-old animals. This finding suggests that $GABA_B$ binding sites in both young adult and aged brain are functionally coupled to G-proteins, confirming previous results in young adults (38) and illustrating the absence of a change in the presence of functional coupling with aging. We do not see any significant changes in the ability for GTP-γ-S to inhibit binding between age groups, a finding that is in agreement with previously reported data (23). However, there may be age-related changes in the types of G-proteins to which $GABA_B$ binding sites are coupled (23).

In conclusion, we find that $GABA_B$ binding decreases significantly from 2 to 23 months in rat brain Binding decreases most dramatically between 2 and 3 months and then levels off, changing very little in the next 2 years. Saturation studies reveal a two-site fit for $GABA_B$ binding in both young and aged animals and the age-related decrease in $GABA_B$ binding appears to be due to an decrease in binding site affinities rather than a decrease in receptor density. This decrease in affinity may result from either a change in receptor-G-protein coupling or a change in the proportion of receptor subtypes with different affinities for GABA. Finally, the pharmacological profiles of $GABA_B$ receptor binding appear to be similar in young and aged animals, suggesting that the functional properties of $GABA_B$ binding are stable throughout adult life.

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