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POSTNATAL ONTOGENY OF GABA$_B$ BINDING IN RAT BRAIN

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Abstract—The postnatal development of GABA$_B$ binding sites in rat brain was studied by quantitative receptor autoradiography using [^{3}H]GABA under selective conditions. Binding levels peak at regionally specific times during the first three weeks of life and then decline to adult levels. GABA$_B$ binding peaked in the globus pallidus, vestibular and spinal trigeminal nuclei, and the CA3 region of the hippocampus at postnatal day 3; in the striatum, nucleus accumbens, inferior olive, septum, dentate gyrus and CA1 region of the hippocampus at postnatal day 7; in the neocortex and thalamus at postnatal day 14; and in the medial geniculate at postnatal day 21. Following these regionally specific peaks, binding decreased to postnatal day 28 levels. Further significant decreases in binding were observed in all regions examined between postnatal day 28 and adulthood. Comparisons of binding site pharmacology reveal equipotent displacement of GABA$_B$ binding by several competitive agonists and antagonists in postnatal day 7 and adult rat brain, indicating that immature and adult binding sites have similar pharmacological properties with regard to these compounds. The GABA$_B$ receptor antagonist CGP 54626A, however, inhibited binding more potently in the postnatal day 7 thalamus and neocortex than in these areas in the adult brain. The guanyl nucleotide analogue guanosine 5'-O-(3-thiotriphasphate) inhibited GABA$_B$ binding extensively in both postnatal day 7 and adult brain. The non-competitive antagonist zinc also inhibited GABA$_B$ binding at both ages and was more potent in postnatal day 7 brain than in adult brain. Saturation analyses reveal two binding sites with similar affinities in both immature and adult rat brain, indicating that postnatal modulation of GABA$_B$ binding reflects changes in binding site density rather than modulation of binding site affinity. While immature GABA$_B$ binding sites share most pharmacological characteristics with adult binding sites and appear to be coupled to G-proteins at an early age, their interactions with zinc and CGP 54626A suggest that GABA$_B$ binding sites in immature brain may have a distinct pharmacological profile.

Our data suggest significant regional and pharmacological changes in GABA$_B$ binding during development. The implications of these findings are discussed with regards to a possible role of GABA$_B$ receptors in the development of the central nervous system.

GABA is the predominant inhibitory neurotransmitter of the CNS. The inhibitory actions of GABA are mediated by two types of receptors, the GABA$_A$ receptor and the GABA$_B$ receptor. While the GABA$_A$ receptor has been extensively described and investigated, the GABA$_B$ receptor is less well understood. In the mature brain, activation of GABA$_B$ receptors can inhibit the release of neurotransmitters[12] and hyperpolarize postsynaptic neurons.[50,51] The activation of GABA$_B$ receptors results in various cellular responses, including alterations in ion channel conductances[11,25] and modulation of intracellular second messenger systems, such as adenylyl cyclase.[32,37,69,70]

A number of studies have revealed modulation of neurotransmitter receptor expression during early postnatal development. Benzodiazepine,[21] kainate,[47] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA),[34] and *N*-methyl-D-aspartate[34,43,63] binding all exhibit regionally specific peaks during the first three postnatal weeks before declining to adult levels. The development of binding to a subtype of glutamate receptor, now known to be a metabotropic receptor,[15] is also modulated in a regionally specific manner. Binding in some regions decreases throughout development, binding in other regions increases, while in some regions binding peaks and then declines to adult levels.[28,38] Immunohistochemical studies have shown developmental modulation of both quantity and distribution of neocortical glycine receptor α-subunits, with a transient peak in immunoreactivity around postnatal day (P) 15.[6] Finally, transiently high levels of GABA$_A$ binding have been observed in various brain stem regions, peaking at or before P10 and then declining to adult levels.[72] In all of these studies, the transient peaks in binding are suggested to indicate a role of these receptors in development.

Our previous results indicate that $GABA_B$ binding follows a unique pattern of development as well. We have observed a peak in $GABA_B$ binding in the cerebellar molecular layer around the end of the second postnatal week, followed by a significant decrease in binding to adult levels. In addition, we observed transient expression of high levels of $GABA_B$ binding in the deep cerebellar nuclei during early postnatal life.[65] While early investigations of G-protein coupling[1] and neocortical electrophysiological responses[39] suggested that $GABA_B$ receptors are not functionally active until the second postnatal week, more recent evidence has shown that presynaptic $GABA_B$ receptor-mediated inhibition is present in somatosensory cortex by P7.[24] Functional postsynaptic $GABA_B$ receptors have also been identified in rat hippocampal slices in the first postnatal week.[16,26,35] In addition to their neurotransmitter function, $GABA_B$ receptors may also modulate neurite outgrowth in the CNS.[46]

In an attempt to more fully characterize the postnatal ontogeny of the $GABA_B$ receptor expression, [³H]GABA receptor autoradiography was used to describe postnatal modulation of $GABA_B$ binding in several forebrain and brainstem structures and to compare the pharmacology of $GABA_B$ receptors in immature (P7) and adult (P42–P52) rat brain.

EXPERIMENTAL PROCEDURES

Materials

[³H]GABA (91.7 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Isoguvacine was purchased from Cambridge Research Chemicals (Cambridge, U.K.). (±)Baclofen was a gift from Dr R. Lovell (Ciba-Geigy, Summit, NJ). 3-Aminopropylphosphonous acid (3-APA; also known as 3-aminopropylphosphinic acid), phaclofen and 2-hydroxysaclofen were purchased from Tocris Neuramin (London, U.K.). CGP 35348 was donated by Drs H. Schroter and L. Maitre (Ciba-Geigy, Basel, Switzerland). CGP 54626A was a gift of Dr S. J. Mickel (Ciba-Geigy, Basel, Switzerland). Guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) was purchased from Sigma (St Louis, MO).

Animals

Adult male Sprague–Dawley rats (P42–P57) and pregnant Sprague–Dawley rats were purchased from Harlan Industries (Indianapolis, IN). For binding studies, male pups were killed one day after birth (P1), P3, P7, P14, P21 and P28. Pharmacology and saturation studies used male pups killed at P7–P8 and adults.

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 or coronal plane) were cut on a Lipshaw cryostat at -20°C and thaw-mounted onto gelatin-coated slides. In the development time course experiments, sections were taken in four coronal planes: (i) the level of the nucleus accumbens; (ii) the level of the globus pallidus; (iii) the level of the thalamus and rostral hippocampus; and (iv) the level of the superior colliculus and medial geniculate (see Fig. 1). Coronal sections at the level of the caudal brainstem were also examined. In the P29 vs adult and pharmacology experiments, brains were cut in the horizontal plane. Sections were stored for no longer than 24 h at -20°C prior to assays.

[³H]GABA quantitative autoradiography

$GABA_B$ binding sites were examined with 20 nM [³H]GABA in the presence of 10 mM isoguvacine.[17] Sections were run in triplicate. 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 [³H]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 2-hydroxysaclofen, 3 μM to 1 mM phaclofen, 30 nM to 1 mM CGP 35348, 30 pM to 1 μM 3-APA, 10 pM to 300 nM CGP 54626A, 10 nM to 100 μM GTP-γ-S and 1 nM to 3 mM zinc were included in the incubation mixture. Saturation experiments were 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 [³H]GABA was used. For concentration points above 33.3 nM, 33.3 nM [³H]GABA was diluted with non-radioactive GABA. Non-specific binding was determined by the addition of 100 μM (±)baclofen. Following incubation, slides were removed individually and rinsed quickly three 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 (³H-Hyperfilm, Amersham) along with standards containing known amounts of radioactivity (ARC, Inc., St Louis, MO) for three to four weeks at $+4$°C. Films were developed for 4 min in Kodak D-19, fixed and dried.

Data analysis

Ligand binding was quantified with computer-assisted densitometry using an MCID system (Imaging Research Inc., St Catherine's, Ont.). 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.[54] Standards were commercial ¹⁴C plastic standards (ARC Inc., St Louis, MO), calibrated against previously described ³H-brain paste standards constructed to give a known amount of radioactivity per mg protein.[54] Optical density readings were taken in regions of

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Fig. 1. Autoradiographs and graphs of [³H]GABA binding to $GABA_B$ binding sites at four coronal levels (see Experimental Procedures for details): level I (A), level II (B), level III (C) and level IV (D), within postnatal ages indicated in the bottom right corner of the autoradiographs. Representative postnatal ages were selected to best illustrate regional peaks in binding. ANOVAs reveal significant differences in $GABA_B$ binding across development in all regions examined ($P < 0.005$). Post hoc analysis with a Scheffe F-test reveals $GABA_B$ binding significantly different from P28 at P1 and P3 in the neocortex, P3, P7 and P14 in the striatum, P3 and P7 in the septum, P1, P3, P7 and P14 in the globus pallidus, P1, P3, P7 and P14 in both CA1 and CA3, P3 and P7 in the dentate, P21 in the medial geniculate, and P14 in the superior colliculus ($P < 0.05$).
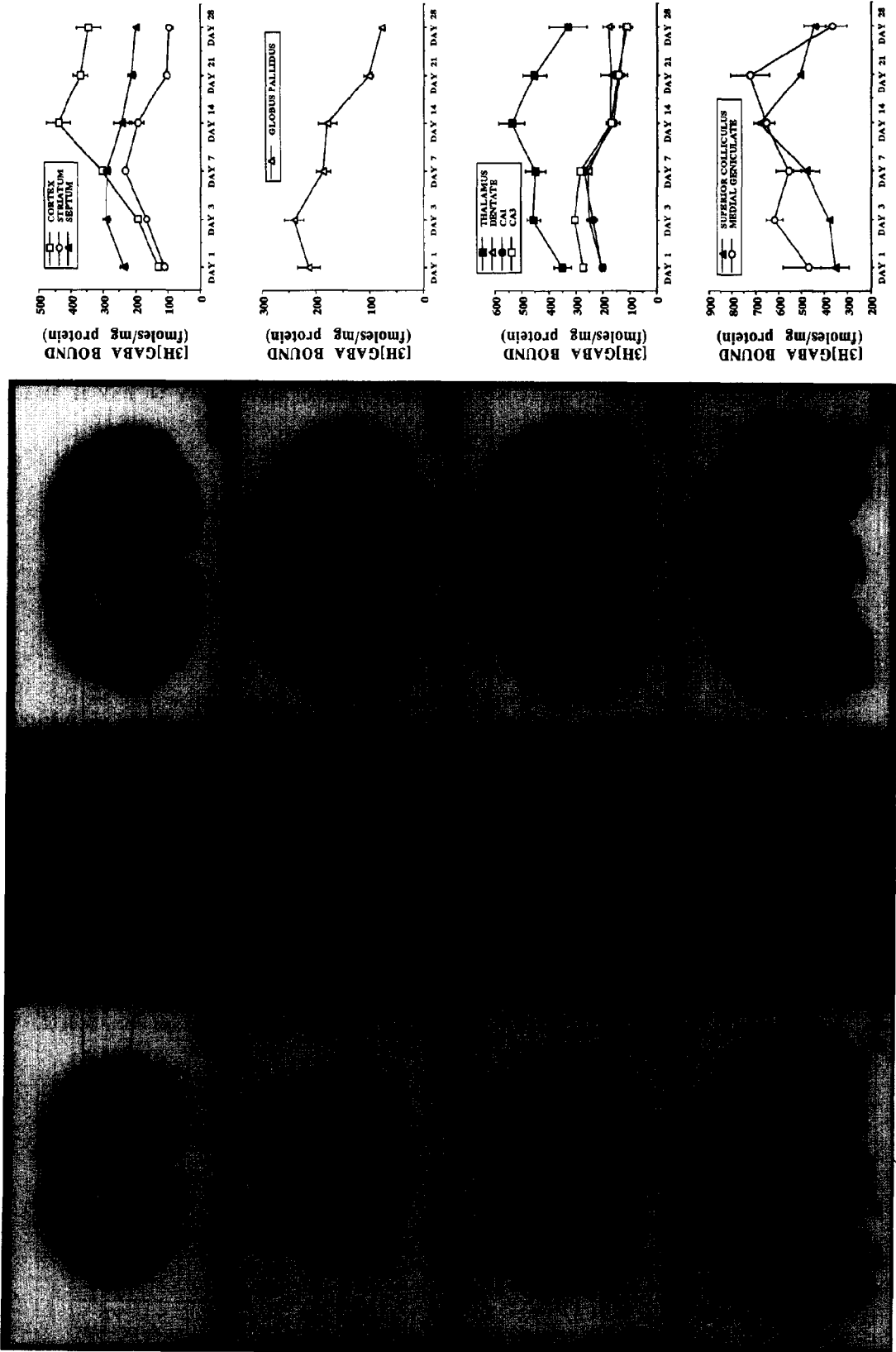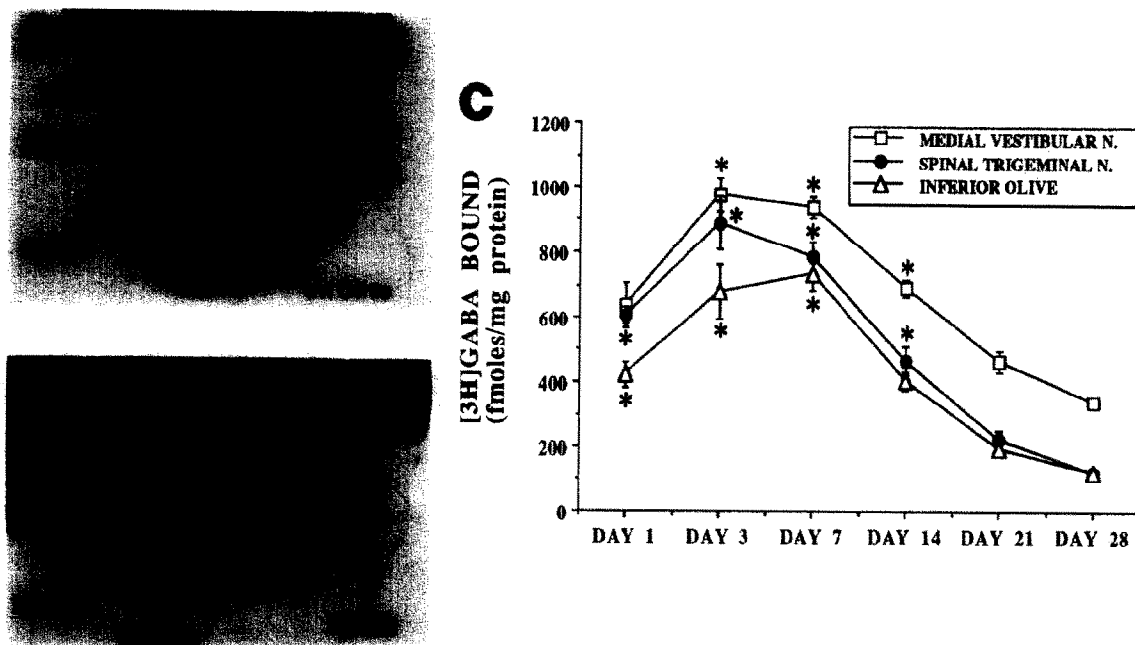
Fig. 1.

Fig. 2. Autoradiographs of [³H]GABA binding to GABA$_B$ binding sites in the medial vestibular nucleus (MVestN), spinal trigeminal nucleus (SpTriN) and inferior olive (Inf Olive) at P3 (A) and P28 (B). Also note extensive binding in the deep cerebellar nuclei (DCN) at P3 which is absent in adult brain.[65] (C) Development of [³H]GABA binding to GABA$_B$ binding sites in the brainstem at P1 ($n = 6$), P3 ($n = 6$), P7 ($n = 6$), P14 ($n = 5$), P21 ($n = 4$) and P28 ($n = 4$). ANOVAs reveal significant differences in GABA$_B$ binding across development in all regions examined ($P < 0.001$). *Post hoc analysis with a Scheffe F-test reveals GABA$_B$ binding significantly different from P28 ($P < 0.05$).

interest and converted to fmol/mg protein values using the standard curve derived from the optical density values of the standards.[54]

Ontogeny. In the first experiment, GABA$_B$ binding in P1–P28 brains was examined. Binding was assessed in the caudal brainstem and in each forebrain structure at the following levels (described above in Tissue preparation): cortex; all levels, striatum; levels I and II, septum and nucleus accumbens; level I, globus pallidus; level II, thalamus and hippocampal regions; level III, and medial geniculate and superior colliculus; level IV. In examining the development of the pattern of binding within the neocortex, only sections taken at level I were analysed.

In the second experiment, GABA$_B$ binding in P28 brains was compared to binding in adult brains.

Pharmacology. IC$_{50}$ values for the displacing ligands were calculated mathematically for four regions using a simple linear regression for points between 10 and 90% of total binding plotted on a dose–response semi-log plot (Cricket Graph, Cricket Software, Malvern, PA). Saturation data were analysed using the computer program LIGAND (Biosoft, Ferguson, MO). LIGAND performed a statistical analysis of the residual variance of one- vs two-site curve fits using a partial F-test. A two-site fit was considered to be preferable at significance levels of $P < 0.05$.

RESULTS

Ontogeny of GABA$_B$ binding

GABA$_B$ binding peaked at regionally specific times during postnatal development. Binding peaked in the globus pallidus and the CA3 region of the hippocampus at P3; in the striatum, nucleus accumbens, inferior olive, septum, dentate gyrus and

CA1 region of the hippocampus at P7; in the neocortex, thalamus and superior colliculus at P14; and in the medial geniculate at P21 (Fig. 1). GABA$_B$ binding peaked in the vestibular and spinal trigeminal nuclei at P3 and in the inferior olive at P7 (Fig. 2). In addition, binding decreased significantly in all regions examined from P28 to adulthood (Table 1).

The distribution of GABA$_B$ binding in the neocortex also changed during development (Fig. 3, Table 2). GABA$_B$ binding was assessed in the outer, middle and inner thirds of the cortex. At P1 and P3, a band of high binding was observed at the outermost part of the cortex, corresponding to cortical layer I. At this age, binding in the middle and inner thirds of

Table 1. Comparison of [³H]GABA binding to GABA$_B$ binding sites in P28 and adult forebrain regions ($n = 3$ at each age)

	P28	Adult
Thalamus	426.5 ± 52.9	255.6 ± 4.2
Neocortex	355.8 ± 30.9	216.5 ± 14.1
Striatum	82.9 ± 7.2	48.9 ± 5.9
Dentate	221.3 ± 21.3	142.2 ± 15.0
CA1	103.9 ± 11.5	60.3 ± 7.1
CA3	82.3 ± 8.4	53.3 ± 5.9

Binding decreased significantly from P28 to adulthood in all areas examined ($P < 0.05$, Student's t-test).
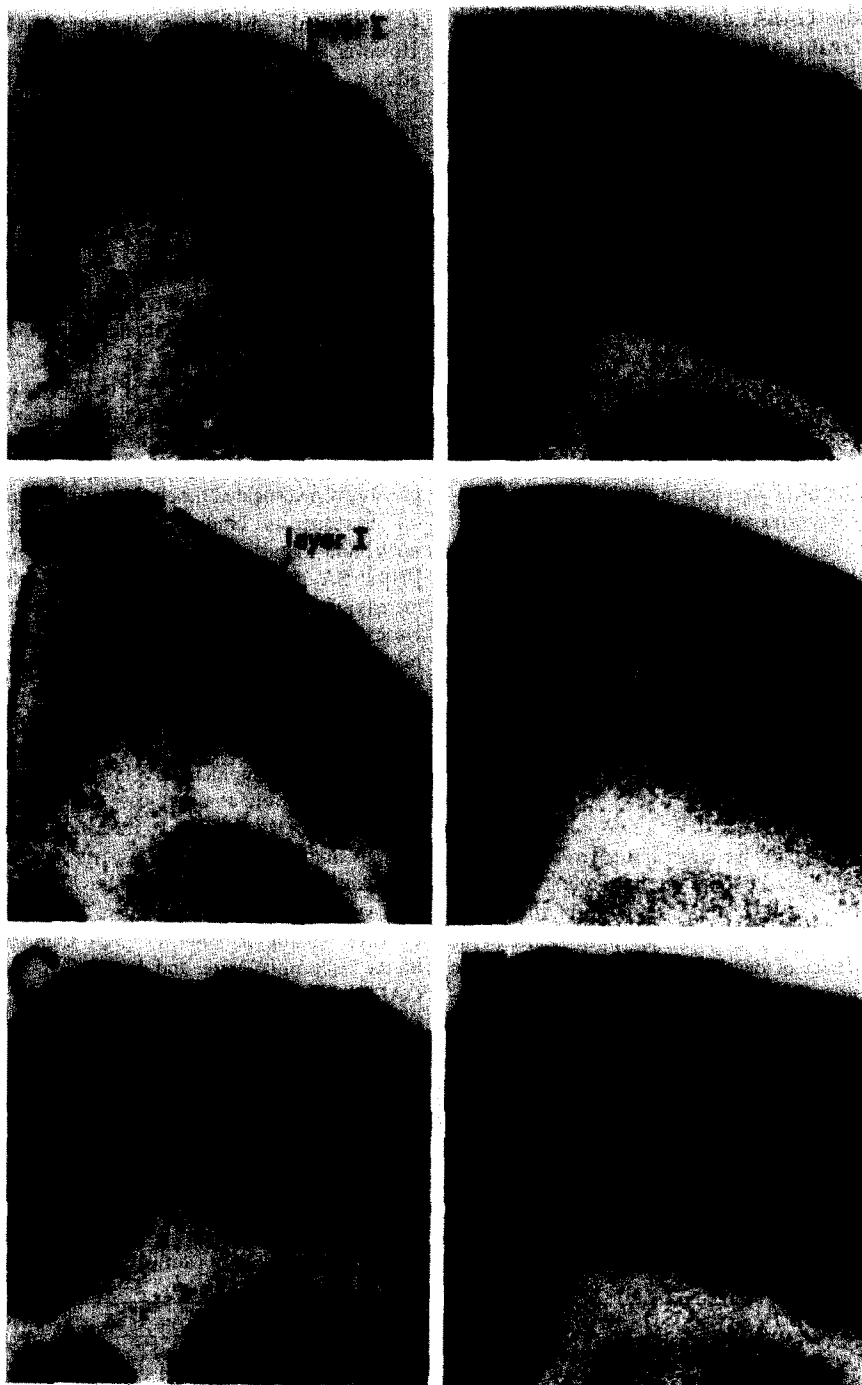
Fig. 3. Autoradiographs of [^{3}H]GABA binding to GABA$_B$ binding sites in the neocortex at P1 (A), P3 (B), P7 (C), P14 (D), P21 (E) and P28 (F). Regional binding levels within the neocortex at these ages are presented in Table 2. Scale bar = 1 mm.

the cortex was greater than binding in the remainder of the outer third, just under the layer I band of high binding. At P7, binding was essentially homogeneous throughout the cortex, with slightly higher levels in the middle third. From P14 on, binding was greatest in the outer third and least in the inner third. These

regional changes were supported by *post hoc* analyses (see Table 2 legend).

Pharmacology of immature vs adult GABA$_B$ binding

Regional IC$_{50}$ values were the same in P7 and adult brain for displacement of GABA$_B$ binding by the

competitive GABA$_B$ agonist 3-APA, and the competitive GABA$_B$ antagonists 2-hydroxysaclofen, CGP 35348 and phaclofen (Table 3). The competition curves for displacement of GABA$_B$ binding by these compounds in P7 and adult brain were virtually superimposable (Fig. 4). The competitive GABA$_B$ antagonist, CGP 54626A,[8,53] also displaced binding equipotently in the cerebellum and hippocampus. However, in the neocortex and thalamus, CGP 54626A inhibited GABA$_B$ binding with significantly greater potency in P7 brain than in adult brain (Fig. 5, Table 3).

Both the guanyl nucleotide analogue GTP-γ-S and the non-competitive antagonist zinc are more potent in immature brain than in adult brain (Fig. 6, Table 3). In addition, the regionally specific enhancement of GABA$_B$ binding by low micromolar concentrations of zinc seen in the cerebellar molecular layer, neocortex and hippocampus of adult brain[64] was not observed in any region of the P7 brain (Fig. 6B).

Saturation studies reveal two affinity sites for both P7 and adult GABA$_B$ binding sites (Fig. 7). K_D and B_{max} values for high- and low-affinity binding sites in the cerebellum, neocortex and thalamus of P7 and adult brain are presented in Table 4.

DISCUSSION

Ontogeny of GABA$_B$ binding

GABA$_B$ binding increases just after birth, peaks at regionally specific times during the first two postnatal weeks and then decreases with adulthood. In interpreting these data, we must address the possible effect of developmental changes in tritium quenching on the observed changes in GABA$_B$ binding. Tritium emissions are absorbed to a greater extent by white matter than gray matter such that increased myelination during development would result in decreased tritium emissions. Therefore, the post-peak decreases in [^{3}H]GABA binding to GABA$_B$ binding sites could theoretically be due

to increased myelination during development. However, decreases in tritium emissions from P14 to adult are of the order of 1–14%,[31] while decreases in GABA$_B$ binding are approximately 40–50%. Thus, the effect of tritium quenching is not great enough to account for the decreases in GABA$_B$ binding observed during development. One region in which tritium quenching does appear to be almost great enough to account for the changes in GABA$_B$ binding seen during development is the globus pallidus.[31] However, previous investigations have shown that binding with [^{14}C]glutamate, the emissions of which are not affected by myelination. gives the same results as binding with [^{3}H]glutamate in the globus pallidus[29] and hippocampus,[43] suggesting that quenching may not explain the decrease in binding we observed in these regions. In addition, we have previously investigated the effect of tritium quenching on decreases in binding in the deep cerebellar nuclei and found that binding with [^{14}C]GABA and [^{3}H]GABA gives the same results.[65] Furthermore, increases in quenching in regions such as the neocortex and thalamus from P5 to P14 suggest that we may be underestimating the magnitude of increases in GABA$_B$ binding from birth to P14 in these regions.

Our findings concerning the changes in GABA$_B$ binding site development are in partial agreement with the little work that has been done in this area. A study of GABA$_B$ binding in the cerebellum, hippocampus and substantia nigra reports no changes in the affinity of GABA$_B$ binding sites for [^{3}H]GABA across development, but higher B_{max} values for GABA$_B$ binding in P14–P17 pups than in adults in the substantia nigra and lower B_{max} values for GABA$_B$ binding in pups in the hippocampus and cerebellum.[27] While we see similar changes in the substantia nigra (unpublished observations), we also see decreased binding in the cerebellum and hippocampus over the course of development. The reason for this discrepancy is not clear. The previous study was done in homogenates and therefore

Table 2. Comparison of [^{3}H]GABA binding to GABA$_B$ binding sites in the neocortex at P1 ($n = 4$), P3 ($n = 4$), P7 ($n = 4$), P14 ($n = 4$), P21 ($n = 4$) and P28 ($n = 3$)

	Layer I	O	M	I
P1	164.4 ± 22.8	127.2 ± 14.8	184.3 ± 9.1	203.0 ± 9.0
P3	289.1 ± 14.3	177.0 ± 10.6	269.5 ± 14.0	275.8 ± 11.1
P7		360.2 ± 9.4	417.4 ± 10.2	376.1 ± 10.8
P14		591.2 ± 70.5	521.5 ± 51.8	416.7 ± 40.3
P21		531.4 ± 21.3	453.7 ± 23.3	345.9 ± 23.7
P28		526.3 ± 47.1	402.4 ± 35.3	303.7 ± 25.4

Binding was analysed in layer I (LI) at P1 and P3, the outer third of the cortex (not including layer I at P1 and P3; O), the middle third of the cortex (M) and the inner third of the cortex (I) as indicated on the autoradiograms in Fig. 3. ANOVAs reveal significant differences in GABA$_B$ binding across the different regions of the neocortex at all ages except P14 ($P < 0.05$). *Post hoc* analysis with a Scheffe *F*-test reveals significant differences in GABA$_B$ binding in LI vs O at P3, O vs M at P3 and P7, M vs I at P21, and O vs I at P1, P3, P21 and P28 ($P < 0.05$).

Table 3. IC_{50} values for inhibition of [³H]GABA binding to GABA_B binding sites by the competitive GABA_B agonist 3-aminopropylphosphonous acid and the competitive GABA_B antagonists 2-hydroxysaclofen, phaclofen, CGP 35348 and CGP 54626A, as well as the guanylyl nucleotide analogue guanosine 5′-O-(3-thiotriphosphate) and the non-competitive GABA_B antagonist zinc

	DCN/ML		Neocortex		Thalamus		Dentate gyrus	
	P7	A	P7	A	P7	A	P7	A
3-APA (nM)	20.0 ± 1.3	24.9 ± 3.6	14.1 ± 4.7	30.0 ± 3.5	10.3 ± 3.6	26.4 ± 1.1	18.9 ± 5.1	35.3 ± 6.6
2-OH-S (µM)	41.3 ± 5.8	52.6 ± 7.7	46.6 ± 11.3	65.1 ± 14.6	62.8 ± 10.8	64.4 ± 13.4	49.6 ± 7.0	53.9 ± 2.7
Phaclofen (µM)	341 ± 53	267 ± 52	470 ± 21	200 ± 46	296 ± 49	297 ± 26	376 ± 58	211 ± 13
CGP 35348 (µM)	33.0 ± 1.5	20.4 ± 2.9	39.9 ± 15.7	32.5 ± 9.7	29.6 ± 10.9	35.6 ± 12.0	38.9 ± 4.0	22.9 ± 6.5
CGP 54626A (nM)	7.7 ± 1.4	8.5 ± 1.4	4.8 ± 0.2**	27.3 ± 2.2	10.9 ± 0.8**	38.3 ± 0.8	6.38 ± 1.0	8.9 ± 0.9
GTP-γ-S (nM)	447 ± 15**	798 ± 53	547 ± 54	1125 ± 235	470 ± 110*	2403 ± 499	499 ± 30**	827 ± 32
Zinc (µM)	312 ± 53**	1140 ± 65	241 ± 13**	884 ± 71	375 ± 82*	1257 ± 183	376 ± 30**	1380 ± 160

Significantly lower IC_{50} values in p7 brain were observed for GTP-γ-S, zinc and, in some regions, CGP 54626A (**$P < 0.01$; *$P < 0.05$). 2-OH-S, 2-hydroxysaclofen; DCN, deep cerebellar nuclei; ML, cerebellar molecular layer.
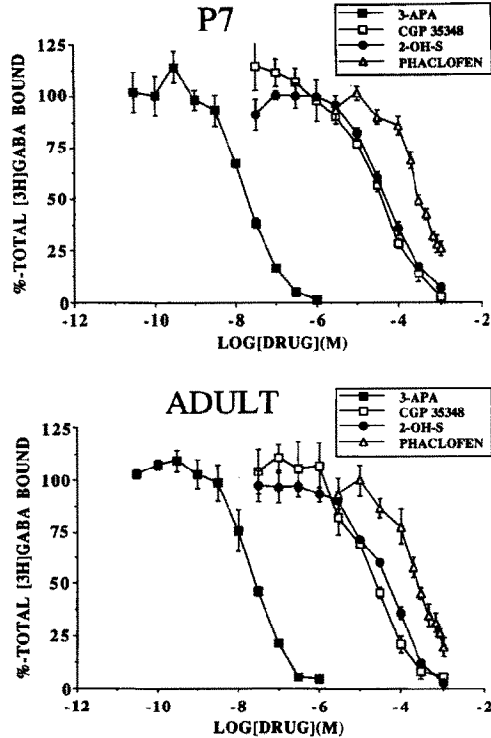

Fig. 4. Inhibition of [³H]GABA binding to P7 and adult GABA_B binding sites by the GABA_B agonist 3-APA and the GABA_B antagonists 2-hydroxysaclofen (2-OH-S), phaclofen and CGP 35348. Binding was evaluated in various forebrain and cerebellar regions ($n = 3$ at each age). Representative curves from the deep cerebellar nuclei of P7 brain and the cerebellar molecular layer of adult brain are shown. These regions were chosen because of the high level of binding and to contrast binding to a purely transient population of binding sites with an adult population. IC_{50} values for other regions are presented in Table 1. No significant differences in IC_{50} values were observed (see Table 1).

methodological differences may have contributed to the observed discrepancies.

Earlier studies concerning developmental changes in GABA receptor binding report increased [³H]GABA binding during development[18] due to a change in receptor number.[2] However, these studies used binding conditions which favour GABA_A binding. A more recent investigation of GABA_A binding using [³H]muscimol receptor autoradiography has indicated that in many brain stem regions, GABA_A binding peaks at or before P10, while binding in other rostral brain regions increases with age and peaks at or after P21.[72] Our results concerning GABA_B binding indicate a similar early peak in the spinal trigeminal nucleus, vestibular nucleus and inferior olive. However, in the other brain regions examined, adult GABA_A binding levels peak around P21 and decline only slightly to adult levels, while GABA_B binding levels peak earlier and decrease significantly to adult levels. The development of GABA_A and GABA_B binding site expression seems to be independent of one another.
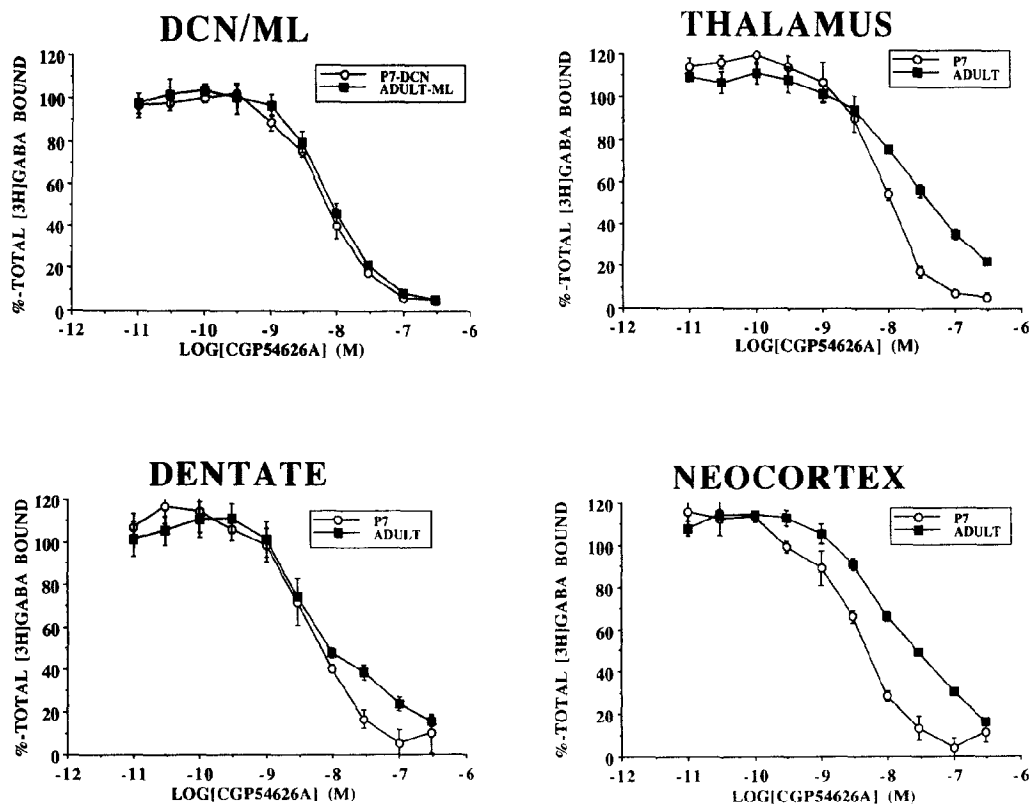
Fig. 5. Inhibition of [³H]GABA binding to GABA$_B$ binding sites by the GABA$_B$ antagonist CGP 54626A in the deep cerebellar nuclei of P7 brain and the cerebellar molecular layer of adult brain (DCN/ML), and the dentate gyrus, thalamus and neocortex of P7 and adult brain ($n = 3$ at each age). IC$_{50}$'s in P7 brain were significantly lower than in adult brain in the neocortex and thalamus ($P < 0.05$; see Table 3).

The ontogeny of GABA$_B$ binding does not seem to correlate with developmental changes in various neurochemical markers of GABAergic synaptic function. Glutamate decarboxylase (GAD), the enzyme mediating GABA synthesis, is detectable in embryonic cortex and increases to adult levels between the third and fourth postnatal weeks.[18,19,60] The development of GABA-transaminase activity, the enzyme mediating GABA catabolism, also increases with development, maintaining a fairly constant GAD/GABA-transaminase ratio throughout development.[66] In addition, GABA levels,[20] high-affinity GABA uptake[18,56] and K$^+$-stimulated Ca^{2+}-dependent GABA release from cortical slices[22] all increase during postnatal development. Unlike these other GABAergic parameters, which exhibit a generalized increase with maturity, GABA$_B$ binding is high at birth, increases to regionally specific peaks and then decreases between P28 and adulthood.

The development of GABA$_B$ binding may correlate with periods of morphological and connectional development in certain regions. In the neocortex, developmental changes in GABA$_B$ binding site distribution seem to coincide with patterns of synaptogenesis. At P1 and P3, a thin band of GABA$_B$ binding

was observed around the outer cortex, corresponding with cortical layer I.[68] From P1 and P4, Blue and Parnavelas[9] found synapses predominantly in layer I and in the subplate region of cortex, corresponding to the areas of greatest GABA$_B$ binding. At P8, approximately when cortical GABA$_B$ binding is more evenly distributed, the concentration of synapses shifts to the more superficial layers and, by P14, the majority of synapses are in the superficial layers, as are the majority of GABA$_B$ binding sites.

Overall, GABA$_B$ binding in the neocortex peaked around the end of the second postnatal week, coinciding with the attainment of adult features in the cortical layers,[23,68] and the refining of corticocortical projections by elimination of axon collaterals/dendrites.[68] P14 also marks the point at which we observed the adult distribution of GABA$_B$ binding, with the highest levels of binding in the outer layers of the cortex. The cytoarchitectonic changes which take place after P14 are reported to be due to a further decrease in cell density due to increases in the neuropil.[23] Therefore, the decreases in GABA$_B$ binding following the peak in binding at P14 could be due to decreased cell density and/or the elimination of axon collaterals or dendrites containing GABA$_B$ receptors.
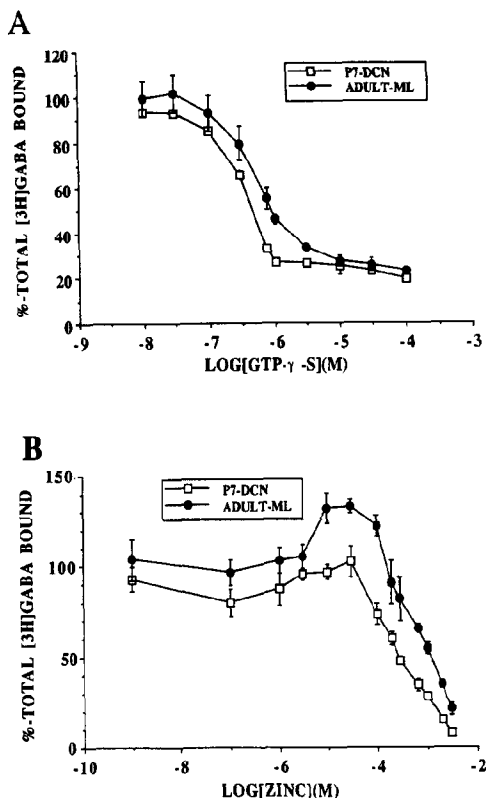
A

B

Fig. 6. Modulation of [³H]GABA binding to GABA_B binding sites by the guanyl nucleotide analogue GTP-γ-S in the deep cerebellar nuclei of P7 brain and the cerebellar molecular layer of adult brain (A) and by the non-competitive GABA_B antagonist zinc in the same regions (B; $n = 3$ at each age). IC_{50} values for both compounds are significantly lower in P7 brain than in adult brain ($P < 0.05$; see Table 3). Note also the robust enhancement of GABA_B binding by lower doses of zinc in the adult brain which is absent in the P7 brain.

GABA_B binding in the thalamus also peaks around P14. We do not know the cellular localization of these binding sites; however, this peak in binding corresponds to the point at which the lemniscal afferents have developed their extensive presynaptic arbors.[57] In addition, the reciprocal projections from the prefrontal cortex to the thalamus appear at P9 and could therefore contribute to the increase in GABA_B binding, should GABA_B binding sites be localized to these elements.[67]

The disappearance of transient projections to and from the thalamus may explain the decrease in GABA_B binding in the thalamus after P14. A contralateral thalamocortical projection has been identified in neonatal rats which undergoes substantial reduction by adulthood.[48] However, neither the precise time course nor the fate of the transiently projecting cells (i.e. cell death, elimination of branches or transformation into local circuit neurons) has been determined. In addition, afferent projections from the dorsal column nuclei, deep

cerebellar nuclei and inferior colliculus are much more extensive at birth than in mature rats.[4] However, only neonatal and mature (P30) rats were examined, so we cannot determine how closely this neuroanatomical change correlates with the change in GABA_B binding in the thalamus.

GABA_B binding in the hippocampus precedes the appearance of many markers of GABAergic synaptic transmission. Significant amounts of GABA_B binding are present in all hippocampal regions at birth, while GAD-positive neurons have been first observed at P4 and GABA-positive neurons do not appear until P6.[59] The early appearance of GABA_B binding sites suggests a non-synaptic role for these sites in the developing hippocampus. The presence of GABA_B binding sites also precedes the earliest observation of GABA_B receptor-mediated responses in the hippocampus, found at P6,[26] and recurrent inhibition involving both GABA_A and GABA_B receptor-mediated responses seen around P5 in CA3 and P9 in CA1.[62] The presence of GABA_B binding sites before the onset of inhibitory neurotransmission in the hippocampus also supports a non-synaptic function for these binding sites in the immature brain.

Evidence is accumulating for the developmental role for GABA in the CNS. The developmental increase in GAD activity precedes the development of GABAergic synapses, suggesting a non-synaptic function for early GABA production.[5,71] GAD has also been localized to growth cone processes in developing rodent cerebellum,[44] suggesting a developmental role for GABA in the immature brain. In addition, recent reports have implicated a synaptogenic action for GABA in the developing brain. The presence of GABA will evoke the development of low-affinity GABA receptors[45] and will increase the number of neurite-extending cells and density of intracellular components[30] in cultured cerebellar granule cells. In vivo, increased extracellular GABA
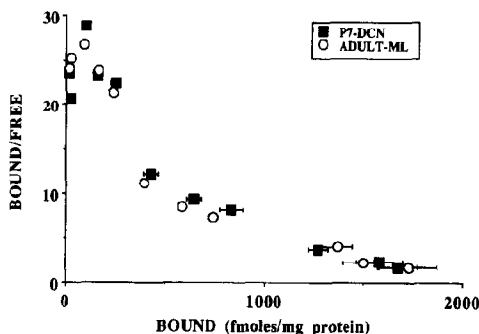
Fig. 7. Saturation analysis of [³H]GABA binding to GABA_B binding sites reveals high- and low-affinity GABA_B binding sites in immature and adult brain ($n = 6$ at each age). A two-site fit was statistically preferred at both ages in all regions examined as determined by a partial F-test using the LIGAND program ($P < 0.001$). Binding was analyzed in the deep cerebellar nuclei of P7 brain and the cerebellar molecular layer of adult brain (curves shown), in the thalamus and in the neocortex.

Table 4. K_D and B_{max} values for high (I) and low (II) affinity $GABA_B$ binding sites in the deep cerebellar nuclei, neocortex and thalamus of P7 rat brain, and the cerebellar molecular layer, neocortex and thalamus of adult rat brain

	DCN/ML		Neocortex		Thalamus	
	P7	Adult	P7	Adult	P7	Adult
K_{DI} (nM)	31.7	16.7	24.4	26.0	25.2	23.0
B_{maxI} (fM)	792	415	299	281	718	400
K_{DII} (nM)	501	478	1120	1050	416	552
B_{maxII} (fM)	1680	1940	1360	2000	1430	1810

DCN, deep cerebellar nuclei; ML, cerebellar molecular layer.

concentrations by nipecotic acid treatment result in increased GABA binding as well.[40,41] However, all of these effects seem to be mediated by $GABA_A$ receptors.

While evidence exists for $GABA_B$ receptor-mediated synaptogenesis in adult tissue,[55] during development $GABA_B$ receptors may mediate inhibition of neurite outgrowth. GABA can increase neurite outgrowth in cultured embryonic chick tectum in serum-containing medium; however, in serum-free medium GABA inhibits neurite outgrowth.[46] GABA may be acting on $GABA_A$ receptors in the presence of serum and on $GABA_B$ receptors in the absence of serum. In addition, the $GABA_B$ agonist baclofen inhibited neurite outgrowth in serum-free medium, while the $GABA_B$ antagonist phaclofen enhanced outgrowth under these conditions.[46]

The presence of high levels of $GABA_B$ binding which precede a fully developed GABAergic system and then decrease into adulthood suggest a role for these receptors in the development of the CNS. In addition, the regionally specific manner in which $GABA_B$ binding peaks in the rat brain suggests a correlation between changes in $GABA_B$ binding and regional developmental profiles.

Pharmacology of immature vs adult $GABA_B$ binding

Most of the competitive $GABA_B$ ligands investigated displace $GABA_B$ binding with approximately the same potency in both immature and adult brain. These results indicate that [³H]GABA binding under these conditions is identifying the same $GABA_B$ binding site in both groups. However, one of the ligands examined, the potent competitive $GABA_B$ antagonist CGP 54626A, displaced binding equipotently in some regions but was significantly more potent in the immature brain at displacing binding in the thalamus and neocortex. Evidence is accumulating for heterogeneity of $GABA_B$ receptors in the CNS.[10,12,14,58] This hypothesis is supported by the discovery of a ligand that displaces $GABA_B$ binding differently in some regions of immature and adult brain and similarly in other regions.

Zinc was also more potent at displacing $GABA_B$ binding in immature brain than in adult brain. Zinc inhibits $GABA_B$ binding in a non-competitive manner in adult brain[64] and may modulate $GABA_B$

receptor function.[73] In addition, zinc has a biphasic effect on $GABA_B$ binding in adult brain, enhancing binding at low doses and inhibiting binding at higher doses (Fig. 7).[64] This effect was not observed in any region in the immature brains. The difference in potency, combined with the absence of low dose zinc-induced enhancement of $GABA_B$ binding in the immature brain, also suggests that early postnatal $GABA_B$ binding sites differ in some respects from the adult binding sites.

GTP-γ-S inhibits $GABA_B$ binding in both young and adult animals, consistent with functional linkage to G-proteins. The observation that GTP-γ-S reduces binding to approximately 30% of total binding suggests that a high percentage of $GABA_B$ binding is in the high-affinity state. Our findings are not in agreement with a previous report of the effects of guanyl nucleotide analogues on $GABA_B$ binding in young and adult brain. Al-Dahan and Thalmann[1] report increased inhibition of $GABA_B$ binding by GTP-γ-S during development, while we see a small but statistically significant decrease in GTP-γ-S inhibition of binding from P7 to adulthood. However, the previous study compared P1 brain to adult brain, used detergent-treated homogenates and used [³H]baclofen instead of [³H]GABA. The discrepancy could be due to methodological differences and/or developmental changes in baclofen binding to the $GABA_B$ receptor.

Two affinity sites for $GABA_B$ binding were observed in both immature and adult brain. K_D values for both sites were essentially the same at both developmental time points, but the B_{max} values for the high-affinity site were slightly higher in immature brain. Slight differences in K_D value for $GABA_B$ binding were observed in the immature deep cerebellar nuclei and the adult cerebellar molecular layer, while K_D values for immature and adult thalamus and neocortex were virtually indistinguishable. Some previous saturation studies reveal a single affinity site with K_D's in the low to mid-nanomolar range.[1,13,17,33,42] While our report of a two-site fit for $GABA_B$ binding is in contrast to the previous finding of a single $GABA_B$ binding site using the same assay,[17] the previous study assessed binding over a considerably smaller range of GABA concentrations. In addition, we have obtained similar results in subsequent studies (unpublished data), as have other studies which

report two affinity states for the $GABA_B$ binding site with similar K_D's to those reported here.[3,52,61] A two-site fit is consistent with the nature of G-protein-coupled receptors.

The observation of regional- and age-dependent differences in $GABA_B$ binding site pharmacology between immature and adult brain suggests the presence of multiple subtypes of $GABA_B$ receptors. Multiple isoforms of glycine,[7] N-methyl-D-aspartate,[36] AMPA,[49] and $GABA_A$ receptors[38] have been discovered which undergo regionally specific changes in their distribution during early postnatal development. Based on our results, we would predict a similar profile for $GABA_B$ receptors.

CONCLUSIONS

Taken together, our data suggest significant ontogenetic changes in the $GABA_B$ receptor. Regionally specific changes in $GABA_B$ receptor binding during development appear to coincide with synaptogenic and organizational events, suggesting a role for $GABA_B$ receptors in the development of the CNS. In addition, the presence of some pharmacological differences between immature and adult $GABA_B$ binding sites suggests that varying proportions of receptor subtypes exist at these two stages of life.

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