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PHARMACOLOGY, DISTRIBUTION, CELLULAR LOCALIZATION, AND DEVELOPMENT OF $GABA_B$ BINDING IN RODENT CEREBELLUM

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Abstract—Quantitative receptor autoradiography using [^{3}H]GABA under selective conditions was used to characterize the pharmacology, distribution, cellular localization, and development of $GABA_B$ binding sites in rodent cerebellum. Pharmacologic analysis of [^{3}H]GABA binding showed that drugs active at $GABA_B$ receptors displaced [^{3}H]GABA with the following order of potency: 3-aminopropylphosphonous acid > CGP 35348 = 2-hydroxysaclofen > phaclofen. GTP-γ-S and GDP-β-S also diminished potently [^{3}H]GABA binding in a dose-dependent manner. The pattern of [^{3}H]GABA binding to $GABA_B$ binding sites was systematically mapped throughout the rat cerebellum. $GABA_B$ binding was greatest in the molecular layer and a pattern of parasagittal zonation was observed in the molecular layer of lobules VII–X in adult rats. The cellular localization of $GABA_B$ binding was investigated using lesion techniques. Neither methyl azoxymethanol lesions of cerebellar granule cells nor 3-acetylpyridine lesions of climbing fibers resulted in a decrease in [^{3}H]GABA binding. Homozygote stumbler mutant mice, deficient in Purkinje cell dendrites, had a significant decrease in [^{3}H]GABA binding in the molecular layer. These results suggest that the majority of cerebellar molecular layer $GABA_B$ binding sites detected by [^{3}H]GABA autoradiography are located on Purkinje cell dendrites. Examination of [^{3}H]GABA binding to $GABA_B$ binding sites during development revealed that binding in the molecular layer peaks between postnatal day 14 and postnatal day 28 and then decreases to adult levels. Transient expression of high levels of $GABA_B$ binding was observed in the deep cerebellar nuclei, peaking at postnatal day 3 and decreasing to adult levels by postnatal day 21.

Our investigation of $GABA_B$ pharmacology yielded data in agreement with previously reported results. We have described a parasagittal pattern of $GABA_B$ binding in the cerebellar molecular layer and assigned the majority of cerebellar $GABA_B$ binding sites to Purkinje cell dendrites. Finally, development studies reveal transient peaks in $GABA_B$ binding in the cerebellar molecular layer and deep cerebellar nuclei.

Two distinct types of receptors mediate synaptic transmission by the inhibitory amino acid neurotransmitter GABA in the central nervous system.[7,9,31,32,38,53] The bicuculline-sensitive $GABA_A$ receptor is part of a chloride ionophore and has additional modulatory sites for benzodiazepines and other compounds.[36,70,82] The bicuculline-insensitive $GABA_B$ receptor is linked to guanosine triphosphate binding proteins[5,28,29,33,54,56,91,99] and is activated by the selective $GABA_B$ receptor agonist baclofen.[7–9,38] $GABA_B$ receptors are located both pre-[9,23,48,87,88] and post-synaptically,[34,73,77–79] as well as on glial cells.[57] Neuronal $GABA_B$ receptor activation can either inhibit Ca^{2+} currents[29,31,56,63,91,100] or activate K^+ currents,[5,41,80,86,90] depending on the cellular localization of the receptor. $GABA_B$ receptor agonists can act through second messenger systems to inhibit basal or forskolin-stimulated cyclic AMP formation,[52,95,97,98] potentiate cyclic AMP formation induced by other neurotransmitters,[52,61,93,95,99] or modulate histamine and serotonin stimulated inositol phospholipid turnover.[20,45] Depending on the brain region examined, $GABA_B$ receptor activation can decrease neurotransmitter release,[9] hyperpolarize postsynaptic neurons,[19,73,77–79] or act presynaptically to inhibit inhibitory neurons.[23,73,74] While the functional roles of the $GABA_B$ receptor are not fully understood, the $GABA_B$ receptor may be involved in the formation of hippocampal long term potentiation,[24,75,76,81] antidepressant action,[59,67,93] nociception,[4,88] epilepsy,[14,74] modulation of neocortical receptor fields,[35] and regulation of thalamic neuronal activity.[21]

We chose to study $GABA_B$ receptors in the cerebellum because the neuroanatomy of the cerebellum is well understood and GABA mediates much of the neural processing within the cerebellum as well as cerebellar cortical output via Purkinje cells. The molecular layer of the cerebellum possesses the second highest density of $GABA_B$ binding sites within the central nervous system.[11,18,96] The cellular localiz-

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Abbreviations: DCN; deep cerebellar nuclei; EAA; excitatory amino acid; GDP-β-S; guanosine 5′-O-(2-thiodiphosphate); GMP; guanosine 5′-monophosphate; GTP-γ-S; guanosine 5′-O-(3-thiotriphosphate); MAM; methyl azoxymethanol; P; postnatal day; Pcp-2; Purkinje cell protein 2; st/+; heterozygous stumbler mice; st/st; homozygous stumbler mice; 3-AP; 3-acerylpyridine; 3-APA; 3-aminopropylphosphonous acid.

ation of $GABA_B$ binding within the molecular layer has not been conclusively established as previous attempts to examine this question have produced conflicting results.[10,62,98] Moreover, $GABA_B$ binding in the molecular layer of the cerebellum follows a pattern of parasagittal zones of alternating high and low density [^{3}H]GABA binding.[1] This study will examine four aspects of $GABA_B$ binding in the cerebellum. We will investigate both the pattern of parasagittal zonation and the cellular localization of $GABA_B$ binding within the molecular layer. We will also describe both the pharmacological characteristics and the postnatal development of $GABA_B$ binding in the rat cerebellum.

EXPERIMENTAL PROCEDURES

Animals

Pharmacology. Adult male Sprague–Dawley (Harlan Industries, Indianapolis, IN) rats weighing 175–195 g were used in the pharmacology experiments. Three animals were used for the agonist/antagonist curves, three for the guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S)/guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S) curves, and three for the guanosine 5′-monophosphate (GMP)/ATP curves.

Mapping. Eight adult male Sprague–Dawley rats weighing 175–195 g were used in mapping experiments. Four brains were cut in the coronal plane and four in the horizontal plane.

Localization studies. Homozygous recessive "stumbler" mutant mice (st/st) were bred from heterozygous parents (st/+) purchased from Jackson Laboratories (Bar Harbor, ME). Stumbler mutants have fewer Purkinje cells and granule cells than normal or heterozygote littermates and the Purkinje cells have an immature dendritic morphology with a reduced number of dendrites and spines.[15] Affected pups were identified by abnormal gait, insufficient weight gain, and loss of the normal righting reflex. Controls were neurologically normal heterozygous littermates (st/+). Two st/st and two control rats were killed at postnatal day (P) 18 and at P 21.

Seven granuloprival rats were produced by injecting male rat pups with methyl azoxymethanol (MAM, 30 mg/kg, diluted to 10 mg/ml in 0.9% NaCl, ip) on P 1. Methyl azoxymethanol is a nucleic acid alkylating agent which kills cells undergoing mitosis at the time of drug administration. When administered on P 1, MAM destroys predominantly granule cells and cerebellar interneurons.[58,60] While MAM does not reduce the number of Purkinje cells,[17] the position of these cells and the orientation and extent of their dendritic arbor may be altered.[13] Six saline injected littermates served as controls. Animals were killed at P 40.

The climbing fiber input to the cerebellum was destroyed in a third group of animals by destroying the inferior olive with 3-acetylpyridine (3-AP) as described by Llinas.[66] Six adult rats were injected with 3-AP (75 mg/kg, i.p.), an antimetabolite which destroys the inferior olivary nucleus,[26,27,66] followed 3 h later by an injection of harmaline (15 mg/kg, i.p.), which causes olivary neurons to fire rapidly, making them more susceptible to 3-AP toxicity.[25,65] 3-AP toxicity was then stopped by an injection of nicotinamide (300 mg/kg, i.p.) 90 min after harmaline treatment.[51,92] All 3-AP treated animals developed an ataxic gait. Controls were six saline injected animals. Animals were killed six days following treatment.

Development studies. Pregnant Sprague–Dawley rats were purchased from Harlan Industries (Indianapolis, IN) and male pups were killed at P 1 ($n = 6$), P 3 ($n = 6$), P 7 ($n = 6$), P 14 ($n = 11$), P 21 ($n = 21$), and P 28 ($n = 11$). Sample sizes in older animals were expanded to include a wider range of anterior to posterior levels. In a separate experiment, binding was compared in three P 28 rats and three adult rats (175–195 g; P 42).

Tissue preparation

Animals were decapitated and their brains were rapidly dissected and frozen in Lipshaw embedding matrix surrounded by powdered dry ice. Cerebella 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. Sections were stored for no longer than 24 h at -20°C.

[^{3}H]*GABA quantitative autoradiography*

$GABA_B$ binding sites were examined with [^{3}H]GABA in the presence of 10 μM isoguvacine.[18] Sections were run in triplicate. Sections were pre-washed 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). 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 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 D-19, fixed, and dried.

Data analysis

Ligand binding was quantitated 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 polynominal regression equation as previously described.[85] 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.[85] 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.[85]

Pharmacology. In the agonist/antagonist experiments, concentrations of 30 nM to 1 mM 2-hydroxysaclofen, 100 nM to 3 mM CGP 35348, 30 μM to 1 mM phaclofen, and 30 pM to 1 μM 3-aminopropylphosphonous acid (3-APA) were included in the incubation mixture. In the G-protein experiments, concentrations of 10 nM to 100 μM GTP-γ-S, GDP-β-S, GMP, or ATP were included in the incubation mixture. $GABA_B$ binding was analysed in the molecular layer of the cerebellum for sections incubated with varying concentrations of 2-hydroxysaclofen, phaclofen, CGP 35348, 3-APA, GTP-γ-S, GDP-β-S, GMP, and ATP. IC_{50} values for each compound were calculated with a standard dose–response semi-log plot. K_I values for agonists and antagonists were calculated according to the equation $K_I = IC_{50}/(1 + [GABA]/K_D)$ using previously established K_D values.[18]

Mapping. Sections were taken every 1 mm throughout the cerebellum in both the horizontal and coronal planes. $GABA_B$ binding was analysed in the molecular layer of the cerebellum. Bands of greater and lesser optical density comprising the pattern of parasagittal zonation were visually identified and analysed to determine the extent of

parasagittal zonation. Photographs were made from the original films and traced onto paper to reconstruct the pattern of parasagittal zonation.

Localization studies. Binding to the molecular and internal granule cell layer of animals in st/st mutant mice, MAM treated rats, and 3-AP treated rats was compared to binding in the respective control groups. Data from experimental and control groups were compared using a Student's *t*-test. Histological analysis of sections from each treatment group was performed on tissue sections fixed over paraformaldehyde vapor and stained with 0.5% Cresyl Violet to ascertain that the lesion treatment had been effective.

Developmental studies. In the first experiment, $GABA_B$ binding in the deep cerebellar nuclei (DCN) and cerebellar cortex was determined in P 1 rats and in the internal granule cell layer, molecular layer, lateral DCN, and medial DCN of P 3, P 7, P 14, P 21, and P 28 rats. In the molecular layer of P 14, P 21, and P 28 rats, total $GABA_B$ binding was determined by averaging optical density readings taken throughout the molecular layer. In a separate analysis, $GABA_B$ binding in the inner molecular layer was determined by sampling the inner third of the molecular layer, near the Purkinje cell layer, and binding in the outer molecular layer was determined by sampling the outer third of the molecular layer, near the external granule cell layer. In addition, readings were taken at P 21 and P 28 of the bands of greater and lesser optical density comprising the pattern of parasagittal zonation within the molecular layer.

In the second experiment, $GABA_B$ binding was compared in the granule cell layer and the molecular layer in P 28 and adult (P 42) animals. In addition, $GABA_B$ binding was measured in the inner and outer molecular layer.

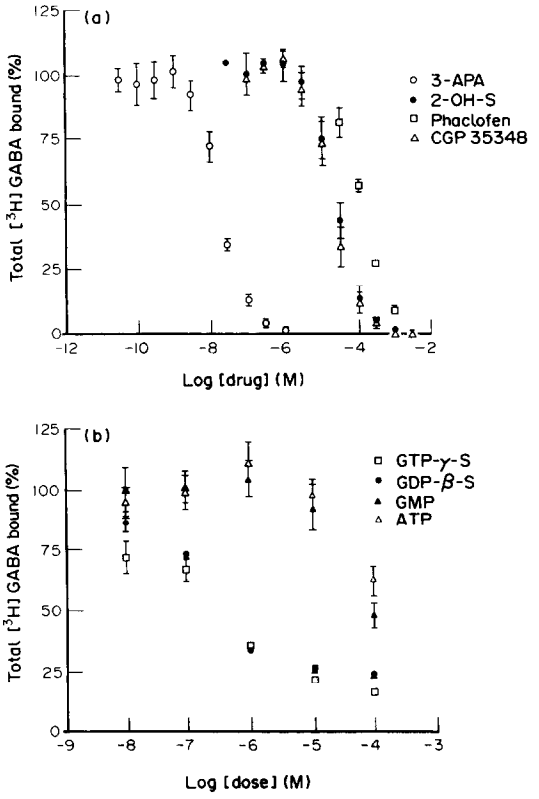

Fig. 1. (A) Inhibition of [^{3}H]GABA binding to $GABA_B$ binding sites in the molecular layer of the cerebellum by the $GABA_B$ agonist 3-APA and the $GABA_B$ antagonists 2-hydroxysaclofen (2-OH-S), phaclofen, and CGP 35348. (B) Inhibition of [^{3}H]GABA binding to $GABA_B$ binding sites in the molecular layer of the cerebellum by GTP-γ-S, GDP-β-S, GMP, and ATP. Error bars represent S.E.M. values.

Table 1. Comparison of IC_{50} and K_I values for various compounds which inhibit [^{3}H]GABA binding to the $GABA_B$ binding site in the molecular layer of rat cerebellum

Ligand	IC_{50}	K_I
3-Aminopropylphosphonous acid (3-APA)	20 nM	19 nM
CGP 34358	25 μM	24 μM
2-Hydroxysaclofen	32 μM	30 μM
Phaclofen	158 μM	152 μM
GTP-γ-S	199 nM	
GDP-β-S	251 nM	
GMP	> 100 μM	
ATP	> 100 μM	

To establish that developmental changes in binding were not due to myelin quenching, [^{14}C] quenching experiments were performed in adult rats using [^{14}C]GABA (150 mCi/mmol). The protocol described above for [^{3}H]GABA experiments was used. Sections were apposed to [^{3}H]Hyperfilm for three months and films were analysed with quantitative densitometry.

Materials

[^{3}H]GABA (91.7 Ci/mmol) and [^{14}C]GABA (150 mCi/mmol) were obtained from Amersham (Arlington Heights, Ill.); isoguvacine was purchased from Cambridge Research Chemicals (Cambridge, England); (±)baclofen was a gift from Dr R. Lovell (Ciba–Geigy, Summit, NJ); 3-APA (also known as 3-aminopropylphosphinic acid), phaclofen, and 2-hydroxysaclofen were purchased from Tocris Neuramin (London); CGP 35348 was donated by Drs H. Schroter and L. Maitre (Ciba-Geigy, Basel, Switzerland); GTP-γ-S, GDP-β-S, GMP, and ATP were purchased from Sigma (St Louis, MO).

RESULTS

Pharmacology

The rank order of potency for displacing [^{3}H]GABA from $GABA_B$ binding sites in the molecular layer was 3-APA $\gg$ 2-hydroxysaclofen = CGP 35348 > phaclofen (Fig. 1A). GTP-γ-S and GDP-β-S decreased the affinity of [^{3}H]GABA for $GABA_B$ sites in the molecular layer with 50% decreases in binding in the nanomolar range while GMP and ATP did not decrease binding by 50% at concentrations up to 100 μM (Fig. 1B). The IC_{50}'s and K_I's for all pharmacology experiments are reported in Table 1.

Mapping

Binding of [^{3}H]GABA to $GABA_B$ sites was greatest in the molecular layer of the cerebellum with an average value of 512 ± 14 fmoles/mg protein (mean ± S.E.M.; all subsequent data are reported as mean ± S.E.M.) while binding in the internal granule cell layer averaged 113 ± 4 fmoles/mg protein. No $GABA_B$ binding sites were found in the adult DCN. Alternating regions of high and low binding formed a pattern of parasagittal zonation in the molecular layer (Fig. 2). Parasagittal zones of high binding averaged 566 ± 64 fmoles/mg while parasagittal zones of lower binding averaged 357 ± 76 fmoles/mg protein ($P < 0.001$, paired *t*-test). Binding in the

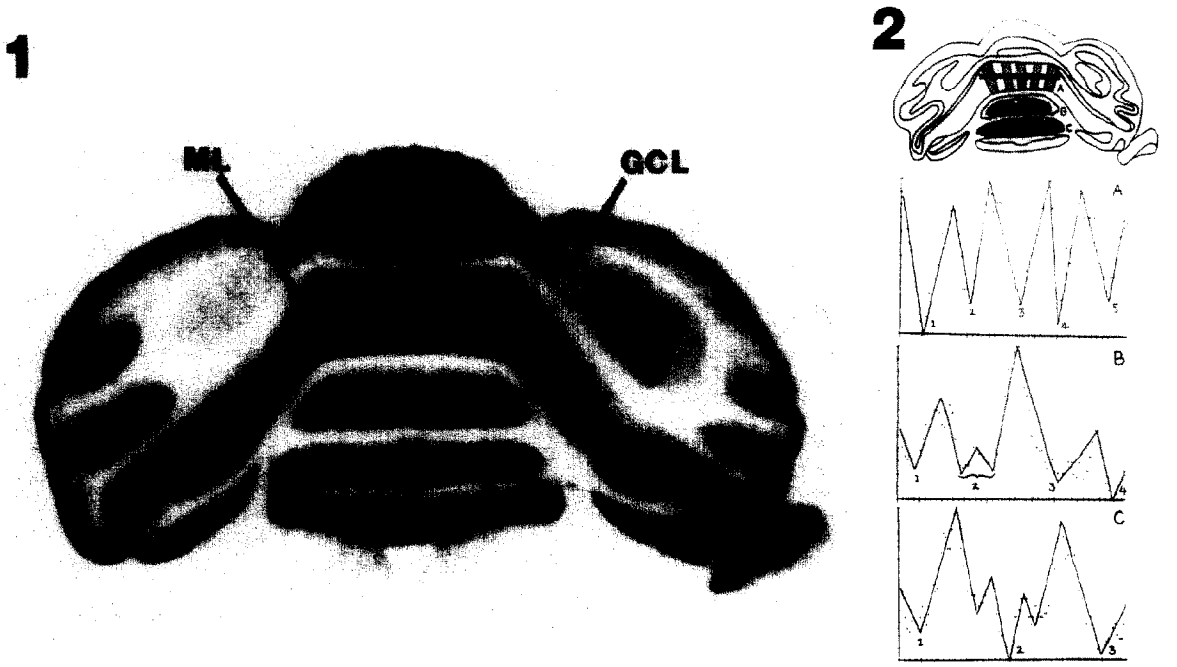

Fig. 2. (1) Autoradiograph of [^{3}H]GABA binding to $GABA_B$ binding sites in the molecular layer of a coronal section of the posterior cerebellar vermis. A parasagittal pattern of [^{3}H]GABA binding is evident composed of five bands of high $GABA_B$ binding in posterior lobule VII and anterior lobule VIII, four bands in posterior lobule VIII and anterior lobule IX, and three bands in posterior lobule IX and anterior lobule X. (2) Histograms A, B, and C indicate the density of [^{3}H]GABA binding along horizontal lines A, B, and C, indicated by the line drawing of the autoradiograph. The horizontal axis represents the line drawn through the image. The vertical axis represents the gray scale value of each pixel, which is proportional to radioactivity level, at each point along the line. Areas of highest density [^{3}H]GABA binding will have the lowest light level with valleys indicating bands of high density [^{3}H]GABA binding.

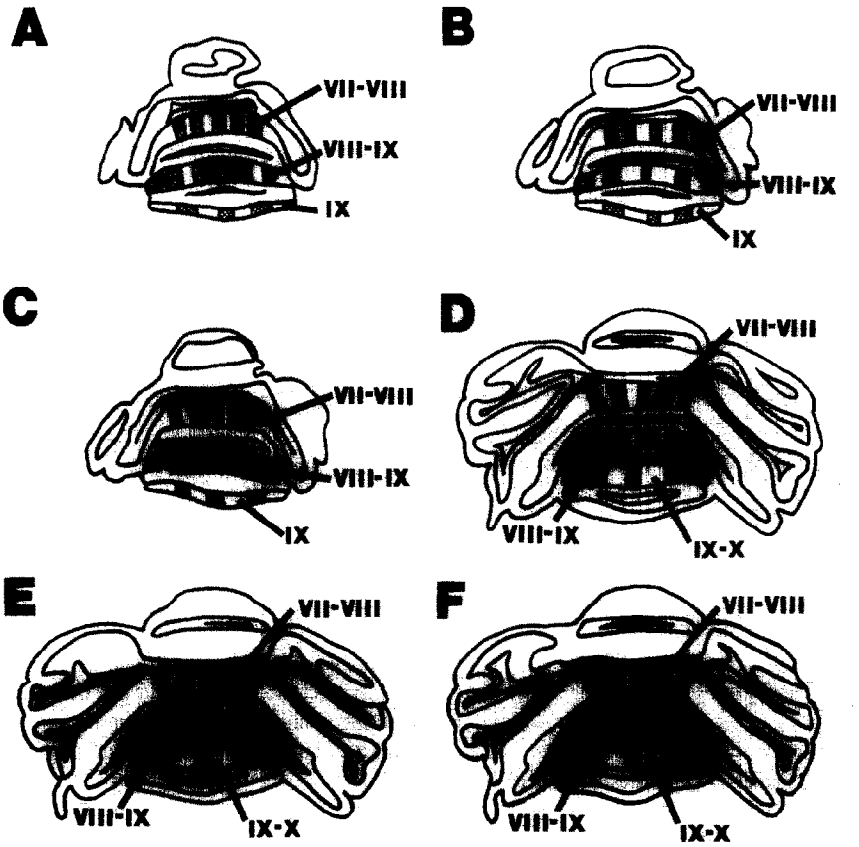

Fig. 3. Reconstruction of the parasagittal pattern of [^{3}H]GABA binding to $GABA_B$ binding sites in coronal sections of the posterior rat cerebellum. Figures represent sections taken every 1 mm from caudal (A) to rostral (F).

parasagittal zones of high binding did not differ significantly from overall binding in the molecular layer. This pattern was found in the posterior vermis, with five bands of high $GABA_B$ binding in posterior lobule VII and anterior lobule VII, four bands in posterior lobule VIII and anterior lobule IX, and three bands in posterior lobule IX and anterior lobule X (Fig. 3). In addition to this parasagittal binding pattern, an anterior to posterior gradient in overall binding levels was observed in the molecular layer in horizontal sections. Binding posterior to the primary fissure averaged 572 ± 24 fmoles/mg protein while binding anterior to the fissure averaged 406 ± 14 fmoles/mg protein ($n = 4$, $P < 0.01$, paired t-test).

Cellular localization of $GABA_B$ binding sites in the molecular layer of the cerebellum

The average binding of [^{3}H]GABA to $GABA_B$ binding sites in the molecular layer of MAM treated rats was 376 ± 29 fmoles/mg protein, which did not differ significantly from MAM control values of 366 ± 8 fmoles/mg protein. Similarly, binding of 398 ± 22 fmoles/mg protein observed in 3-AP treated animals was not significantly different from 3-AP control binding of 349 ± 16 fmoles/mg protein. The average $GABA_B$ binding level in the molecular layer of stumbler mutant mice was 306 ± 22 fmoles/mg protein which was significantly decreased as compared to neurologically normal littermates with

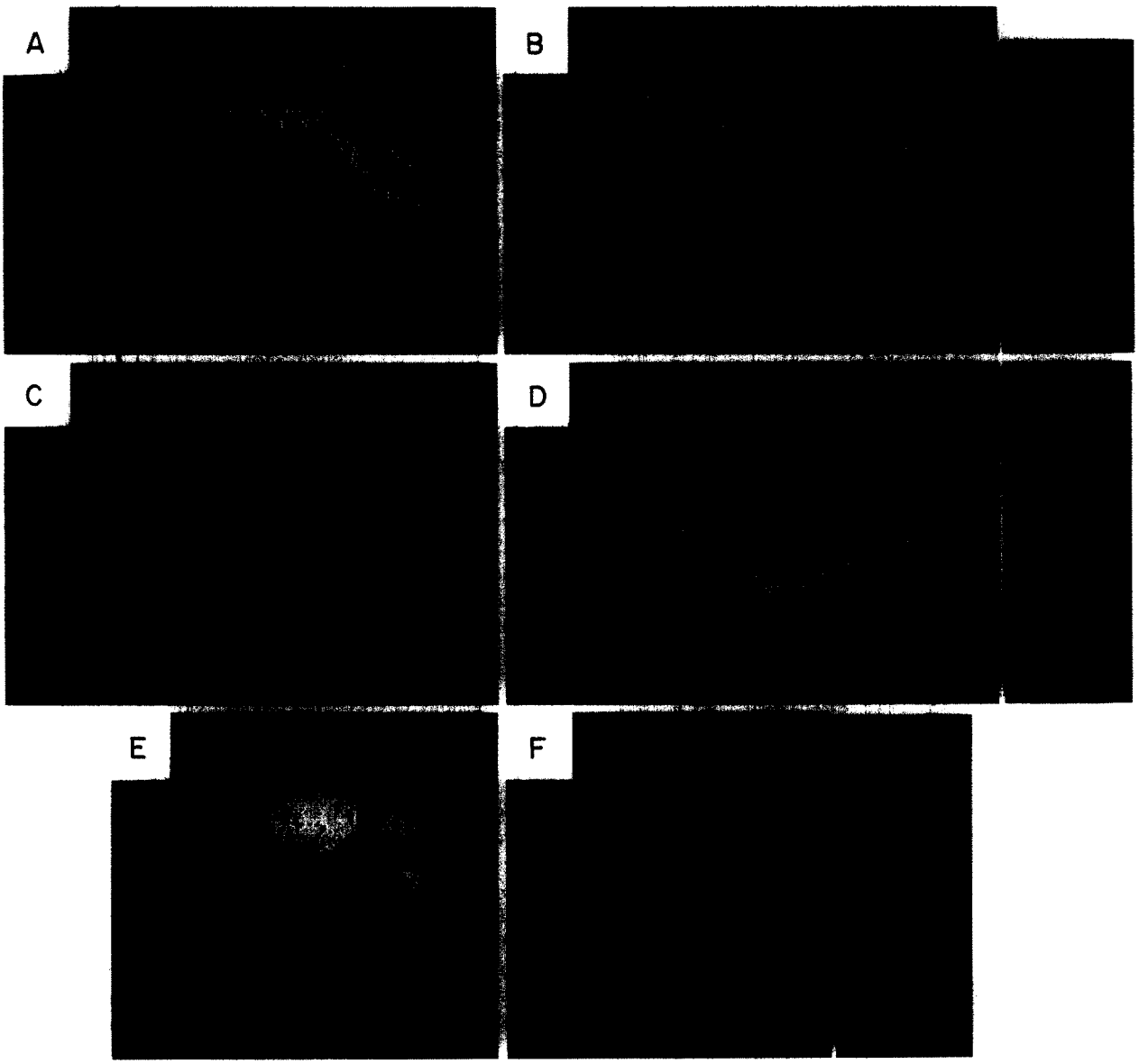

Fig. 4. Autoradiographs of [^{3}H]GABA binding to $GABA_B$ binding sites in coronal sections of the cerebella of (A) MAM control rats, (B) MAM treated rats, (C) 3-AP control rats, (D) 3-AP treated rats, (E) st/+ mice, and (F) st/st mice. Color bars to the right indicate the color scale for each pair of animals.

an average binding level of 550 ± 11 fmoles/mg protein (Figs 4, 5, $P < 0.001$, Student's t-test). No differences were found between control and experimental groups for $GABA_B$ binding levels in the internal granule cell layer (data not shown); however, the low level of $GABA_B$ binding sites in the internal granule cell layer makes $GABA_B$ binding an insensitive measure of changes in the internal granule cell layer. Histology revealed that MAM animals had the gyrated folia typical of MAM treated animals[55] and that 3-AP treated animals had profound loss of inferior olive neurons.

Development of $GABA_B$ binding in rat cerebellum

Deep cerebellar nuclei. $GABA_B$ binding in the DCN was transient (Figs 6 and 7). Binding levels peaked at P 3 with an average value of 833.3 fmoles/mg protein and gradually decreased to adult levels by P 21. Binding in the medial DCN decreased more rapidly with maturity than in the lateral DCN. Paired t-tests revealed that this difference was significant at P 3 ($P < 0.05$), P 7 ($P < 0.0001$), P 14 ($P < 0.001$), and P 21 ($P < 0.005$). Quenching experiments revealed that when [^{14}C]GABA was used in place of [^{3}H]GABA, abundant binding was observed in the cerebellar molecular layer but DCN binding was indistinguishable from background levels.

Molecular layer. $GABA_B$ binding in the molecular layer was discernible by P 3 and increased with maturity, peaking between P 14 and P 28 (Figs 6, 7). Comparisons of P 28 and adult (P 42) binding revealed a 25% decrease in $GABA_B$ binding between P 28 (501 ± 56 fmoles/mg protein) and adult (376 ± 8 fmoles/mg protein) rats ($P < 0.05$, one-tailed t-test). A gradient of binding was observed within the molecular layer at P 14 and P 21. $GABA_B$ binding in the inner molecular layer was $29 \pm 1\%$ higher than binding in the outer molecular layer at P 14 and $13 \pm 1\%$ higher in the inner molecular layer than in the outer molecular layer at P 21 ($P > 0.001$, two-tailed paired t-test; Fig. 8). In P 28 and adult (P 42) animals there was no difference in $GABA_B$ binding between the inner and outer molecular layer. Parasagittal zonation of $GABA_B$ binding was not visible at P 1, P 3, P 7, or P 14. By P 21, parasagittal zonation could be detected (Fig. 8) and the magnitude of the difference between the zones of low $GABA_B$ binding and those of high binding increased with maturity. At P 21 and P 28, binding in zones of lower binding was 19% and 25% lower, respectively, than in zones of higher binding. Results from the mapping study revealed that in adults, binding in low binding zones was 37% lower than in higher binding zones (see above). The presence of lower binding zones at P 21 and P 28 does not decrease overall binding levels in the molecular layer because these bands comprise a fraction of the entire molecular layer.

Internal granule cell layer. Binding to the $GABA_B$ site in the internal granule cell layer was also discernable by P 3, peaked between P 14 and P 28 (Figs 6, 7), and then declined 48% ($P < 0.05$, two-tailed t-test) by adulthood (P 42).

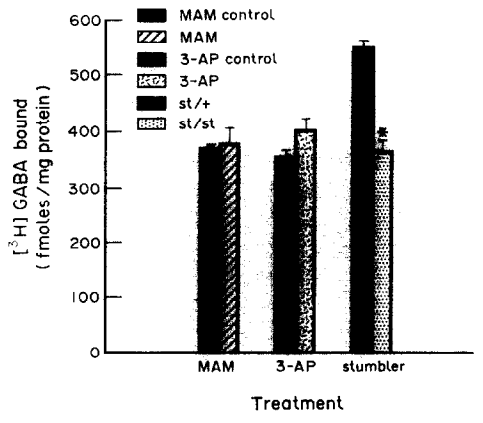

Fig. 5. [^{3}H]GABA binding to $GABA_B$ binding sites in lesioned and control animals. MAM and 3-AP treated rats are compared to saline injected MAM and 3-AP controls and stumbler mutant mice are compared to neurologically normal heterozygous littermates. Error bars represent S.E.M. values. Binding in MAM and 3-AP treated animals did not differ significantly from their respective controls. Binding in st/st mutants was significantly lower than in st/+ littermates. *($P < 0.001$).

DISCUSSION

Pharmacological characteristics of $GABA_B$ binding in rat brain

The rank order of potency of agonists and antagonists observed in this study concur with previously reported values in homogenate and functional studies.[6,30] Differences in the absolute values of K_i's reported in this study as compared to others may be due to differences in technique. For example, autoradiography preparations cannot be washed as thoroughly as homogenate preparations, preventing the complete removal of endogenous GABA. Endogenous GABA will complete with the labeled ligand and alter K_i values. However, despite these discrepancies, the rank order of potency of these ligands as well as the approximate differences in magnitude between K_i's are the same as those previously reported in homogenate studies. In addition, the guanyl nucleotide analogues GTP-γ-S and GDP-β-S potently decreased $GABA_B$ binding while GMP and ATP did not. The inhibition of $GABA_B$ binding by GTP-γ-S and GDP-β-S is consistent with a G-protein coupled receptor. Our results concerning the relative potencies of these agonists and antagonists and the effect of guanyl nucleotide analogues on [^{3}H]GABA binding are in agreement with previously reported data concerning $GABA_B$ binding,[6,30,54] confirming that [^{3}H]GABA is binding to $GABA_B$ binding sites in our assay.

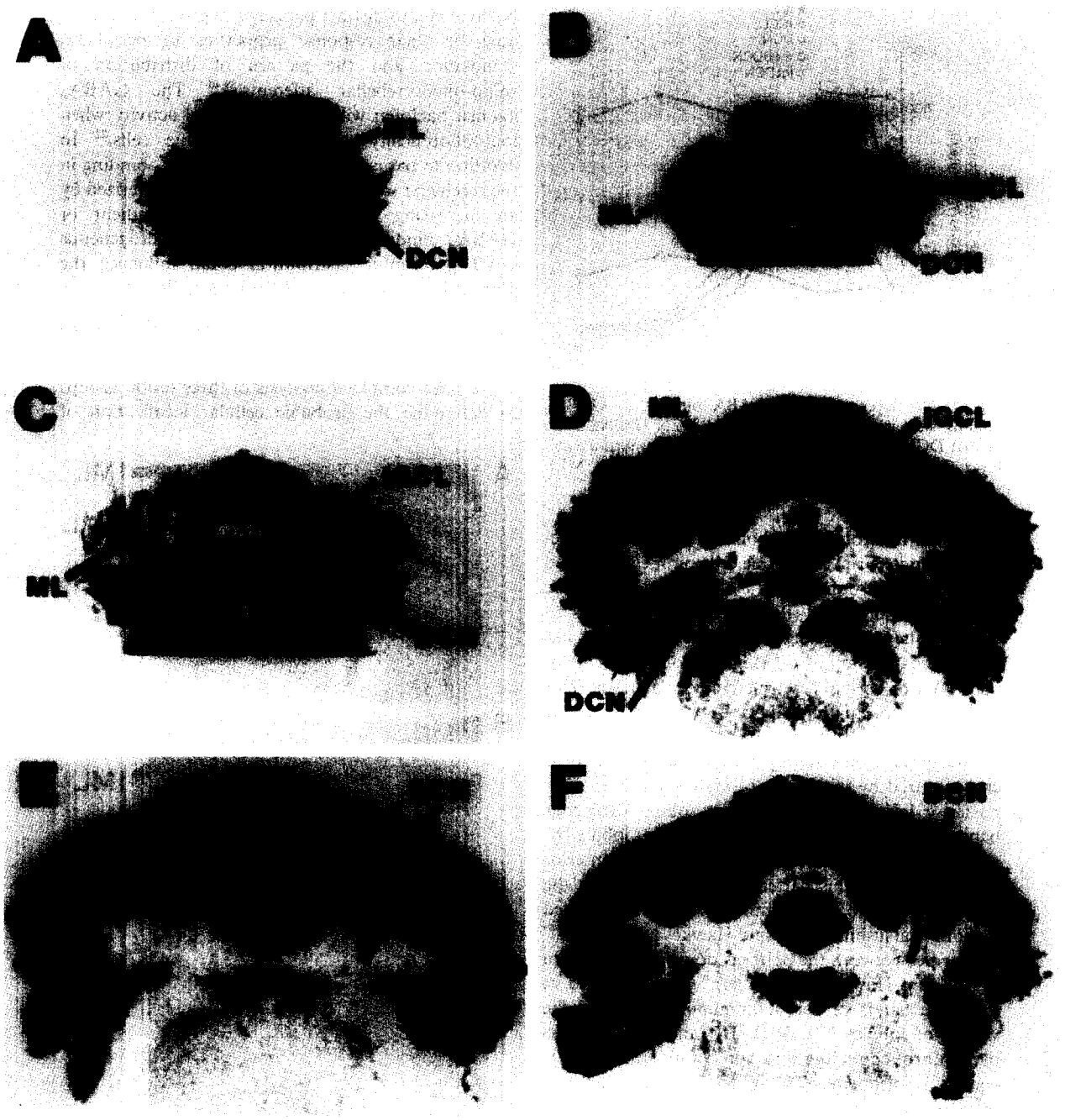

Fig. 6. Autoradiographs of [^{3}H]GABA binding to $GABA_B$ binding sites in coronal cerebellar sections at (A) P 1, (B) P 3, (C) P 7, (D) P 14, (E) P 21, and (F) P 28. Binding reaches adult levels in the molecular layer (ML) and the internal granule cell layer (IGCL) by P 14. Note the transient expression of $GABA_B$ binding sites in the DCN, decreasing in the medial DCN more quickly than the lateral DCN and reaching adult levels by P 28. Sections shown were taken at an anterior level to illustrate transient expression of $GABA_B$ binding sites in the DCN. This level does not show parasagittal zonation.

Mapping of $GABA_B$ binding sites in the cerebellum

Our mapping results confirm the original observation by Albin and Gilman[1] that there are parasagittal zones with high and low levels of $GABA_B$ binding in the molecular layer of the cerebellum. A number of neuroanatomical markers in the cerebellar cortex are also organized in a parasagittal fashion. In the molecular layer, climbing fibers terminate in a pattern of parasagittal zonation according to the location of climbing fiber origin within the inferior olive.[47] Other markers follow a pattern of parasagittal zonation identical to that of climbing fiber termination patterns. These include the

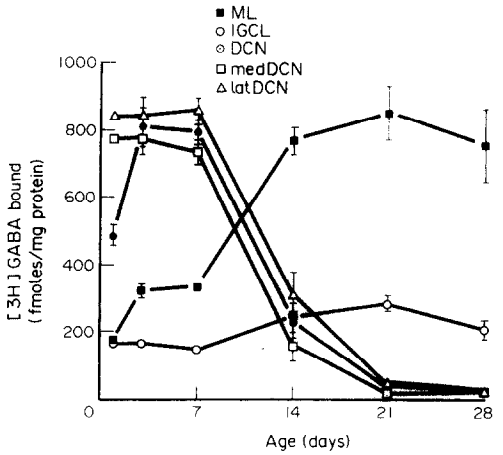

Fig. 7. Development of [^{3}H]GABA binding to $GABA_B$ binding sites in the DCN, the lateral DCN (DCNL), the medial DCN (DCNM), the cerebellar internal granule cell layer (IGCL), and the cerebellar molecular layer (ML). Error bars represent S.E.M. values.

adenosine nucleotide metabolizing enzyme 5′-nucleotidase, and zebrin, a Purkinje cell epitope recognized by monoclonal antisera.[37,47,49,71] In addition, the Purkinje cell protein 2 (Pcp-2) gene, when expressed with specific flanking regulatory DNA elements, has localized expression in parasagittal compartments of the posterior vermal cerebellar molecular layer. This expressed Pcp-2 pattern appears to be similar to the distribution of zebrin immunoreactivity.[94]

The pattern of $GABA_B$ binding reported here is characterized by five bands of high binding levels in posterior lobule VII and anterior lobule VIII, four bands in posterior lobule VIII and anterior lobule IX, and three bands in posterior lobule IX and anterior lobule X. While this pattern is not the same as the banding pattern of zebrin immunoreactivity observed by both Matsushita *et al.*[71] and Hawkes and Leclerc,[49] similarities between the patterns do exist. Matsushita *et al.*[71] and Hawkes and Leclerc[49] have reported at least five bands of zebrin immunoreactivity in the vermis of lobules VII, VIII, IX, and X as well as bands throughout the anterior vermis and the cerebellar hemispheres. The zebrin immunoreactivity pattern resembles the pattern of $GABA_B$ binding in posterior lobule VII and anterior lobule VIII; however, we see fewer bands in other posterior lobules. The presence of fewer parasagittal bands of $GABA_B$ binding than seen with zebrin immunoreactivity might be explained by the poorer anatomical resolution of receptor autoradiography as compared to immunohistochemistry. On the other hand, parasagittal $GABA_B$ binding site zones may only label a subset of the parasagittal zones identified with other markers. Further studies will be necessary to clarify the relationship between $GABA_B$ parasagittal zonation and other anatomic markers of parasagittal zonation.

Cerebellar cortical functions may also be organized into parasagittal zones. Electrophysiological studies have demonstrated that cerebellar Purkinje cells can be divided into distinct parasagittal groups differentiated by their response properties to peripheral stimulation and the pattern of distribution of spino-olivocerebellar afferents.[39,40] The $GABA_B$ agonist baclofen will inhibit neuronal activity when iontophoretically applied to Purkinje cells.[22] In addition to parasagittal zonation, $GABA_B$ binding in the cerebellar molecular layer is also characterized by an increasing anterior to posterior gradient of $GABA_B$ binding. Both aspects of heterogeneous $GABA_B$ receptor distribution could influence the physiological properties of Purkinje cells.

Cellular localization of $GABA_B$ binding in the molecular layer of the cerebellum

We have combined analysis of three lesion groups to determine the probable cellular localization of

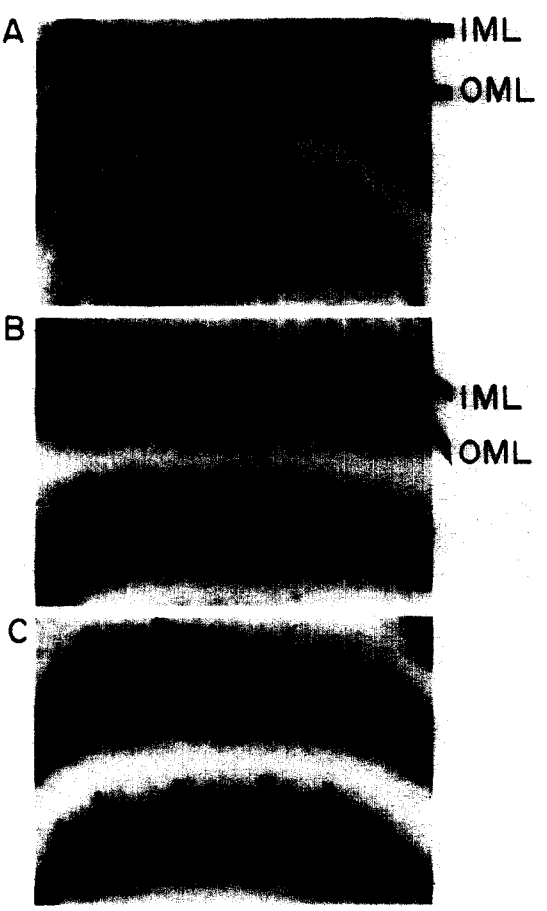

Fig. 8. Autoradiographs of [^{3}H]GABA binding to $GABA_B$ binding sites in coronal sections of the posterior vermis at (A) P 14, (B) P 21, and (C) P 28. Posterior lobule VII (top) through anterior lobule IX (bottom) are pictured. Note the appearance of bands of high $GABA_B$ binding (*) at P 21. The pattern becomes more distinct by P 28 and begins to resemble adult patterns (see Fig. 2). A gradient of binding in the molecular layer was quantified with computer-assisted densitometry at P 14 and P 21 with greater binding in the inner molecular layer (IML) near the Purkinje cell layer and less binding in the outer molecular layer (OML). The lines indicate the approximate position of the IML and OML. Scale bar = 0.8 mm.

$GABA_B$ binding sites within the molecular layer of adult rodent cerebellar cortex. Previous localization studies have yielded conflicting results.[10,62,98] It has been suggested that molecular layer $GABA_B$ binding sites may be located on parallel fibers,[98] on the terminals of climbing fibers,[62] or on cerebellar Purkinje cell dendrites.[10] Our data support the conclusion that the majority of $GABA_B$ binding sites are located on Purkinje cell dendrites. Neither 3-AP treatment, which destroys the olivocerebellar climbing fibers, nor MAM treatment, which predominantly depletes granule, stellate, and basket cells,[58,60] produced a significant decrease in [^{3}H]GABA binding to $GABA_B$ sites in the molecular layer of the cerebellum. Stumbler mutant mice, however, known to have decreased numbers of both granule cells and Purkinje cells as well as immature Purkinje cell dendritic arbors, displayed significantly less $GABA_B$ binding in the molecular layer than controls.

While this type of lesion analysis must be interpreted cautiously because secondary changes occur in neuronal populations spared by the toxic or genetic lesion,[13,17,89] lesion and mutant studies are a historically useful method for determining the cellular localization of neurotransmitter binding sites in the cerebellum.[46,50,68,83] The decrease in $GABA_B$ binding in homozygote stumbler mutant mice could be due to either decreased parallel fibers or Purkinje cell dendrites. The absence of decreased $GABA_B$ binding in MAM treated rats, whose primary deficiency is in the granule cell population, however, suggests that the majority of $GABA_B$ binding sites detected by our assay are not located on the parallel fibers. Our conclusions assume identical neuronal distribution of $GABA_B$ binding in the cerebella of rats and mice.

Our suggestion that $GABA_B$ receptors are located on Purkinje cell dendrites is supported by other data. Bowery *et al.* examined several strains of mutant mice with primary deficits in different neuronal populations to conclude that $GABA_B$ receptors are located primarily on Purkinje cell dendrites.[12] In addition, electrophysiological studies have revealed that iontophoretically applied baclofen inhibits Purkinje cell activity.[22]

The discrepancy between our findings and previous results may be due to the different methods used to measure $GABA_B$ receptors in the previous studies. Kato *et al.* measured [^{3}H]baclofen binding in homogenate preparations and reported decreases in $GABA_B$ binding followed 3-AP treatment.[62] Wojcik *et al.* cited decreases in $GABA_B$ receptor-mediated inhibition of adenylate cyclase activity in Weaver mutant mice as evidence for concluding that $GABA_B$ receptors were located on parallel fibers.[98] While the Weaver mice are deficient in granule cells, they are also secondarily deficient in Purkinje cell dendrites,[89] which could be the source of the observed reduction in $GABA_B$ activity. However, Wojcik *et al.* also reported the absence of any reduction of $GABA_B$ receptor-mediated inhibition of adenylate cyclase activity in Nervous mutant mice which are deficient in Purkinje cells.[98]

While the results obtained by Wojcik *et al.*[98] do not concur with our findings, this may be due to either the indirect method of assessing the presence of $GABA_B$ receptors in the previous study or to the presence of presynaptic $GABA_B$ binding sites which our experiments were unable to detect. It has been shown that the great majority of cerebellar molecular layer adenylate cyclase is localized in the parallel fibers.[50] the localization of adenylate cyclase to granule cell terminals suggests that $GABA_B$ receptor-mediated inhibition of adenylate cyclase might be a sensitive measure when used to detect $GABA_B$ receptors on granule cell terminals but would not be particularly sensitive to changes in $GABA_B$ receptors on Purkinje cells. Another possible source of variance between our results and those of Wojcik *et al.*[98] is the shrinkage of the molecular layer in MAM treated rats. Shrinkage could obscure decreases in $GABA_B$ binding in the MAM treated group which might result from the presence of presynaptic $GABA_B$ receptors on parallel fibers. Shrinkage will also cause underestimation of the decreases in binding in st/st mutant mice.

Recent immunohistochemical studies with an antibody to L-baclofen indicate that $GABA_B$ receptors in the cerebellar molecular layer are found on both Purkinje cell dendrites and parallel fibers.[69] The immunohistochemical data, combined with previous evidence for $GABA_B$ receptor localization on both Purkinje cells and parallel fibers, suggest that cerebellar molecular layer $GABA_B$ receptors are located on both cell types. However, our data indicate that most cerebellar molecular layer $GABA_B$ binding sites detected by [^{3}H]GABA autoradiography are located on Purkinje cell dendrites.

Development of $GABA_B$ binding in the cerebellum

The most striking feature of cerebellar $GABA_B$ binding site development is a robust transient expression of $GABA_B$ binding in the deep cerebellar nuclei. This binding did not completely decrease to adult levels until P 21. This developmental decrease in binding levels could theoretically result from decreased tritium emissions in adult animals due to increased myelination of the deep cerebellar white matter. Tritium emissions are absorbed to a greater degree by white matter than gray matter.[44] If tritium "quenching" due to increased myelination were a factor in the postnatal decrease in DCN $GABA_B$ binding, then binding assays using [^{14}C]GABA in adults should reveal increased DCN $GABA_B$ binding when compared with [^{3}H]GABA binding studies. Such an increase was not observed and white matter quenching cannot be the cause of transient $GABA_B$ binding in the DCN.

Our data also indicate that $GABA_B$ binding peaks between P 14 and P 28 in the molecular layer before

decreasing to adult levels. This result is interesting as neurogenesis and the formation of synaptic connections within the molecular layer is ongoing between P 14 and P 28. In addition, previous studies have indicated that neither $GABA_A$ nor excitatory amino acid (EAA) binding sites in the cerebellar cortex reach adult levels until around P 20 or later.[43,84] While overall binding in the molecular layer peaked by P 14, a developmentally immature pattern of binding is present. $GABA_B$ binding in the lower molecular layer (adjacent to the Purkinje cell layer) is significantly higher than binding in the upper molecular layer (subjacent to the external granule cell layer) in P 14 and P 21 animals. While we cannot be sure of the neuronal localization or function of these $GABA_B$ binding sites in immature animals, this gradient of binding in the molecular layer parallels both the formation of basket and stellate cells in the molecular layer and the appearance of synaptic contacts between parallel fibers and Purkinje cell dendrites. Basket cells are first seen around day 6–7 in the lower molecular layer. Newly formed basket and stellate cells are then stacked from the bottom of the molecular layer (our inner molecular layer) upward, with mature stellate cells appearing at day 21 and later.[2] Synaptogenesis between parallel fibers and Purkinje cell dendrites also follows a progression from the bottom of the molecular layer upwards. In day 15 animals, synapses are beginning to form in the lower part of the molecular layer. By day 21, the Purkinje cells appear mature, but parallel fiber synapses have yet to be formed in the upper molecular layer. Finally, the upward progression of synaptogenesis seems to be complete by 30 days.[3] However, while $GABA_B$ binding is greater in the bottom of the molecular layer at days 14 and 21, there is substantial $GABA_B$ binding in the upper molecular layer.

The pattern of parasagittal zonation is not visible at P 14, begins to appear at P 21, and resembles mature patterns by P 28. The development of parasagittal zones of $GABA_B$ binding is similar to the ontogeny of zebrin immunoreactivity. Leclerc *et al.* reported that zebrin immunoreactivity is first apparent at P 6 and all Purkinje cells are zebrin immunoreactive by P 12.[64] By P 15, suppression occurs in a subset of Purkinje cells, making a parasagittal pattern of zebrin+ and zebrin− Purkinje cells. The pattern of zebrin+ and zebrin− zones does not reach adult appearance until P 30. Our data suggest that the appearance of the parasagittal $GABA_B$ binding pattern also results from a decrease in $GABA_B$ binding in zones destined to exhibit low levels of binding in adults, as zones of lower binding had lower binding levels than the rest of the molecular layer while zones of higher binding had approximately the same level of binding seen throughout the molecular layer. In addition, the magnitude of differences between high and low binding level zones increased with maturity. The inability to detect patterns of parasagittal zonation of $GABA_B$ binding sites prior to P 21 might also be due to the limited anatomical resolution afforded by autoradiographic methods as compared to immunohistochemistry.

Postnatal modulation of cerebellar neurotransmitter sites is not restricted to $GABA_B$ binding. Transient postnatal expression of EAA binding sites which decrease to adult levels by P 25 has also been demonstrated in mouse DCN.[42] It has been suggested that these transiently expressed EAA binding sites may have a developmental function rather than a conventional synaptic receptor function. In addition, changing pharmacological characteristics of EAA binding sites[42,43] and alterations in associated strychnine-insensitive glycine binding[16] have been demonstrated in cerebellar cortex during early postnatal development. The transient expression of $GABA_B$ binding in the DCN and the early presence of high $GABA_B$ binding levels in the molecular layer suggest that $GABA_B$ receptors may play a role in the development of the cerebellum. A role in development is supported by evidence that $GABA_B$ receptors can mediate developmental effects. *In vitro* studies indicate that baclofen can inhibit neurite outgrowth in cultures of embryonic chick tectal neurons while phaclofen, a $GABA_B$ antagonist, will stimulate neurite outgrowth under these conditions.[72]

CONCLUSION

The pharmacology and distribution of $GABA_B$ binding sites in the cerebellum are in agreement with previously reported studies. The observation of a parasagittal zonation pattern in the molecular layer has been confirmed. This pattern is characterized by five bands of higher binding in the molecular layer of the vermis in the posterior lobule VII and anterior lobule VIII, four bands in posterior lobule VIII and anterior lobule IX, and three bands in posterior lobule IX and anterior lobule X and may be a subset of previously defined markers of cerebellar cortical zonation. An anterior to posterior gradient of increasing $GABA_B$ binding was also noted. Localization studies indicate that the majority of the cerebellar molecular layer $GABA_B$ binding sites detected with our assay are located on Purkinje cell dendrites. Developmental studies indicate that $GABA_B$ binding in the molecular and granular cell layer of the cerebellum peaks between P 14 and P 28 and then decreases to adult levels. Transient expression of $GABA_B$ binding sites in the deep cerebellar nuclei which decreases to adult levels by P 21 was also observed. Further studies are needed to address the functional nature of these $GABA_B$ binding sites at different stages of development.

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