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The Development of Type I and Type II Benzodiazepine Receptors in the Mouse Cortex and Cerebellum

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GARRETT, K. M. AND B. TABAKOFF. *The development of Type I and Type II benzodiazepine receptors in the mouse cortex and cerebellum.* PHARMACOL BIOCHEM BEHAV 22(6) 985–992, 1985.—The postnatal development of benzodiazepine (BDZ) receptors was monitored in Heterogeneous Stock (HS) mice, and the BDZ receptors were characterized and categorized into Type I and Type II receptors. When the number of ^{3}H-Flu binding sites (B_{max}) was assessed at weekly intervals after the birth of the animal, the number of sites in both the cortex and cerebellum increased significantly if the data was expressed as fmol/mg tissue. On the other hand, no significant change in ^{3}H-Flu binding sites was evidenced in the cortex, and the number of ^{3}H-Flu binding sites in the cerebellum decreased during postnatal development if B_{max} values were expressed as fmole/mg protein. When receptor binding data was analyzed for the presence of Type I and Type II BDZ receptors, the changes in K_D values for ^{3}H-Flu binding during development could be accounted for by changes in relative proportions of Type I and Type II receptors present in the cortex and cerebellum during the maturation process. Type II receptors predominated in both cortex and cerebellum at birth, and Type I receptors proliferated primarily during the first two weeks of postnatal life. In the cortex of adult mice there were approximately equal numbers of Type I and Type II BDZ receptors. In the cerebellum of adult mice, computer assisted analysis of binding data could not distinguish the presence of two distinct BDZ binding sites. However, Hill coefficients and overall binding constants determined from data on CL-218,872 displacement of ^{3}H-Flu binding to cerebellar membranes indicated that cerebellar tissue from adult mice did contain a heterogeneous array of BDZ receptors.

Benzodiazepine receptor CL-218,872 Cerebellum Cortex ^{3}H-flunitrazepam Receptor ontogeny

THE benzodiazepines (BDZs) are generally classified as anxiolytic drugs, but these drugs produce a variety of additional pharmacological effects. All the actions of the BDZs have been suggested to be initiated by their binding to distinct receptors in the CNS [15,24]. The discovery of multiple BDZ binding sites in the CNS led to the hypothesis that particular receptor subtypes may mediate specific pharmacological effects of the BDZs [8,9]. The BDZ receptor subtypes have been classified as Type I and Type II BDZ receptors, based on the findings of preferential binding of the β-carbolines [3] and the trizolopyridazine, CL-218,872, to CNS membrane binding sites [8]. Lippa *et al.* [9] have proposed that the binding of the BDZs to the Type I receptor mediates anxiolytic and anticonvulsant effects and the binding of the BDZs to the Type II receptor produces the sedative and ataxic effects of these drugs.

Although the postnatal development of Type I and Type II BDZ receptors has been investigated using rat brain tissue [6,10], the prior studies did not examine the kinetic characteristics of individual receptor subtypes during development and little data is available on postnatal BDZ receptor development in animals other than the rat [18]. Our present study examined the postnatal development of the BDZ receptors in Heterogeneous Stock (HS) mice, and used a weighted, least-squares, curve-fitting analysis [16] to distinguish and characterize the kinetic constants for the Type I and Type II BDZ receptors during postnatal development of the animals.

METHOD

Animals

Heterogeneous stock (HS) mice were mated [7] and offspring of both sexes were sacrificed postnatally at weekly intervals for five weeks. Adult animals used in our studies were approximately 15 weeks of age.

Preparation of Synaptic Membranes

Whole brains were quickly removed from the sacrificed mice, and the cortex and cerebellum were dissected and

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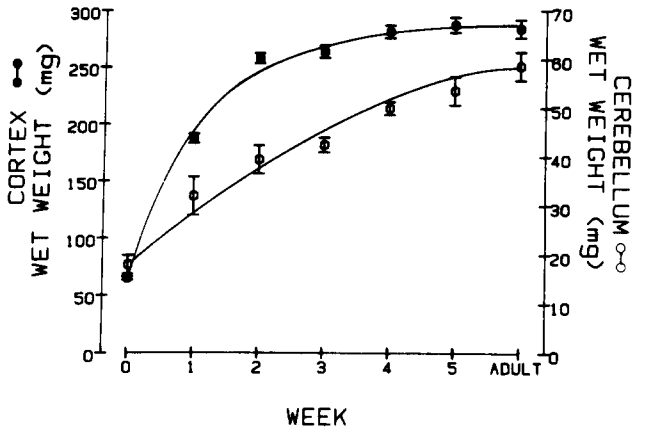

FIG. 1. Development of mouse cortex and cerebellum. The cortex (●—●) and cerebellum (○—○) were dissected and weighed. The values are the mean±SEM of brain regions from four to nine experiments, with each experiment containing 3–15 animals.

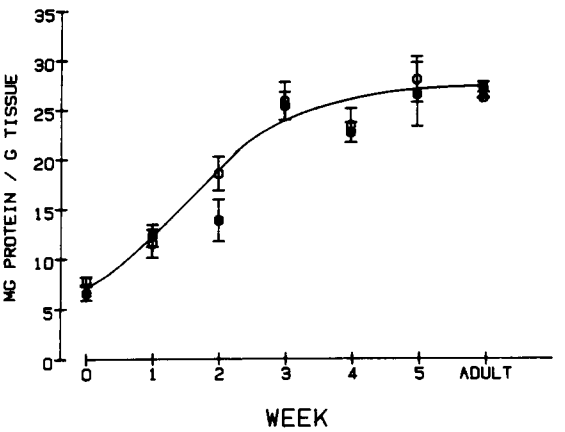

FIG. 2. Membrane protein concentrations in cortical and cerebellar membranes. The concentration of the membrane protein recovered per weight of cortical (●—●) and cerebellar (○—○) tissue was determined at weekly intervals. The values are mean±SEM from four to nine experiments, with each experiment containing 3–15 animals.

weighed. The brain regions were homogenized in 20 volumes of 0.32 M sucrose and 5 mM HEPES (pH 7.4), using a glass-teflon homogenizer. The homogenate was centrifuged at 1,000 × g for ten minutes. The pellet was discarded and the supernatant was centrifuged at 48,000 × g for 20 minutes. The resulting pellet was suspended in distilled water and dispersed using a Brinkman Polytron. The suspension was centrifuged at 48,000 × g for 20 minutes and the resultant pellet was washed five times with cold buffer (100 mM NaCl, 50 mM Na^+/K^+ PO_4; pH 7.4) to remove endogenous ligands [23,25]. The final recovered pellet was resuspended in sufficient buffer to give a protein concentration of 0.2–0.5 mg protein/ml.

^{3}H-Flunitrazepam and CL-218,872 Binding

BDZ receptor binding characteristics were measured by incubating 0.7 ml aliquots of the membrane suspension at 0–4°C with nine separate concentrations of ^{3}H-Flu (0.2–15 nM) in a total volume of 1 ml. Non-specific binding at each ^{3}H-Flu concentration was determined by the addition of 10 μM diazepam to equivalent incubation mixtures. After 75 minutes, duplicate aliquots (0.25 ml) of each of the incubation mixtures were filtered on Whatman GF/B filters and the filters were washed twice with 5 ml of cold buffer. The filters were dried and placed in vials containing 12 ml of scintillation fluid and radioactivity was measured by a Beckman scintillation counter. Displacement of ^{3}H-Flu with CL-218,872 was studied by incubating 0.7 ml of membrane suspension with 1 nM ^{3}H-Flu and varying concentrations of CL-218,872 (20–20,000 nM). The mixture was incubated at 0–4°C for 75 minutes and processed for quantitation of bound radioactive material as described above.

Protein

Membrane protein concentrations were measured by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Analysis of Results

The K_D and B_{max} values for binding of ^{3}H-Flu were calculated using Eadie-Hofstee analysis. The regression lines were fitted to data points by unweighted least-squares analysis. Data from displacement experiments were analyzed using a computerized, weighted, least-squares curve-fitting program, LIGAND [16]. Analysis of variance was used to determine whether the data fit a one- or two-site model, and statistical significance was designated as $p<0.05$. The calculations of K_D values for CL-218,872 took into account the changes in the overall K_D values of ^{3}H-Flu at each age.

Materials

^{3}H-Flu (76.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Sucrose and HEPES were obtained from Sigma (St. Louis, MO). All other buffer salts were obtained from Fisher Scientific (Pittsburgh, PA). CL-218,872 was generously donated by L. R. Meyerson (American Cyanamid, Pearl River, NY).

RESULTS

The weights of the cortex and the cerebellum were measured at weekly intervals after birth. The cortex weighed 65 mg at birth (Week 0), and the weight increased over the first two weeks after birth to equal the weight of the adult cortex (Fig. 1). The cerebellum weighed 18 mg at birth; the weight increased with age and reached adult values five weeks after birth (Fig. 1). The amount of membrane protein obtained after the centrifugation and washing procedure from cortical and cerebellar tissue at varying ages is shown in Fig. 2. The yield of membrane protein per mg (wet weight) tissue increased with age at similar rates in the cortex and cerebellum and reached adult values by the third week after birth.

The saturation binding of ^{3}H-Flu to the BDZ receptor can be used to determine the total number of central BDZ (Type I plus Type II) receptors [8]. Binding of ^{3}H-Flu was examined in the cortex and cerebellum at weekly intervals after birth to determine the overall ontogeny of BDZ binding sites and the kinetic characteristics of ^{3}H-Flu binding.

Eadie-Hofstee analysis of ^{3}H-Flu binding in the cerebral

TABLE 1

DEVELOPMENT OF ^{3}H-FLU RECEPTORS IN THE CORTEX AND CEREBELLUM

Week	Cortex B_{max} fmole/mg tissue	Cortex B_{max} fmole/mg protein	Cerebellum B_{max} fmole/mg tissue	Cerebellum B_{max} fmole/mg protein
0	10.9 ± 1.03 (8)	1,970 ± 99 (9)	17.01 ± 1.39 (5)	2,026 ± 90 (5)
1	18.2 ± 1.12 (6)	2,143 ± 137 (6)	13.21 ± 2.15 (3)	1,442 ± 161 (3)
2	37.9 ± 2.15 (6)	2,056 ± 36 (5)	20.51 ± 1.86 (3)	1,023 ± 116 (3)
3	57.8 ± 4.50 (9)	2,303 ± 162 (9)	32.46 ± 1.67 (5)	1,375 ± 135 (5)
4	60.5 ± 2.64 (5)	2,231 ± 205 (9)	33.93 ± 1.24 (4)	1,351 ± 160 (4)
5	51.3 ± 4.25 (5)	2,085 ± 59 (5)	41.84 ± 3.57 (3)	1,512 ± 157 (3)
Adult	61.9 ± 1.05 (4)	2,138 ± 111 (9)	38.82 ± 2.98 (3)	1,576 ± 178 (4)

^{3}H-Flu binding to cortical and cerebellar membranes was examined postnatally at weekly intervals. The number of binding sites (B_{max}) was expressed as either fmole receptor per mg tissue or fmole receptor per mg protein. Each value represents the mean ± SEM of three to nine experiments and each experiment contained tissue from 3–15 animals. Data expressed as either fmole/mg tissue or as fmole/mg protein was analyzed by statistical methods listed in the legend to Fig. 3. See text for results of statistical analysis.

cortex revealed that the K_D for ^{3}H-Flu at the BDZ receptors at birth (1.8 nM) was lower ($p<0.05$) than the K_D determined with tissue from the adult animals (2.2 nM) (Fig. 3). When the K_D values were compared at various times after birth we found that one week after birth, the K_D for the cortical BDZ receptors decreased ($p<0.005$) as compared to the value obtained at day 0 (Fig. 3) and then the K_D values increased progressively to adult values by three to four weeks of age. The K_D for ^{3}H-Flu at the cerebellar BDZ receptors at birth (1.6 nM) was lower ($p<0.005$) than the K_D in the adult mice (2.9 nM). The dissociation constant (K_D) remained unchanged for the first week after birth, increased thereafter, and reached the adult values when the mice were four weeks old (Fig. 3). Due to developmental changes in protein content of brain membranes (Fig. 2), the number of BDZ receptors in cortex and cerebellum in our study was expressed both on the basis of fmoles/mg tissue (wet weight) and fmoles/mg protein (Table 1). The number of cortical BDZ receptors (B_{max} expressed as fmole/mg tissue) at birth was 18% of the number of receptors in the cortex of adult mice. After birth, the receptor number increased (week one value compared to birth value, $p<0.005$; week two value compared to week one value, $p<0.005$) and reached adult values by three weeks of age (Table 1). The cerebellar BDZ receptor number at birth (fmoles/mg tissue) was 44% of the adult value. The cerebellar BDZ receptor number expressed per mg tissue decreased slightly at one week after birth ($p<0.05$), and then rose progressively to the adult level by three weeks of age.

The development of the BDZ receptor shows a different profile when data is expressed in terms of fmoles of receptor/mg protein. The number of cortical BDZ receptors remained constant during brain development when the data was expressed per mg of protein (Table 1). The cerebellar BDZ receptor number expressed as fmole/mg protein, decreased during the initial week after birth (week one value compared to birth value, $p<0.01$) the trend was reversed during the third week after birth and the B_{max} increased to the adult level by five weeks of age (Table 1). When the B_{max} for cerebellar BDZ receptors was expressed on the basis of fmole/mg protein the adult values remained significantly below the B_{max} values noted at birth ($p<0.025$).

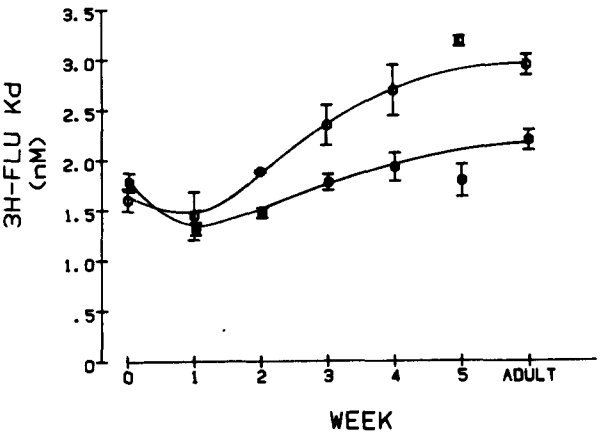

FIG. 3. Development of BDZ receptor affinity for ^{3}H-Flu in the cortex (●—●) and cerebellum (○—○). BDZ binding was assayed postnatally at weekly intervals in the cortex and cerebellum. The concentration of ^{3}H-Flu was varied from 0.2–15 nM, and the ^{3}H-Flu dissociation constant (K_D) was determined by Eadie-Hofstee analysis. Each point represents a mean ± SEM of three to nine experiments. Each experiment contained tissue from 3–15 animals. Data for cortical and cerebellar tissue was subjected separately to analysis of variance and the Student *t*-test was used, *post-hoc*, to characterize differences between mean values obtained at various times during development. See text for results of statistical analysis.

Type I and Type II BDZ receptor subtypes can be differentiated by examining the displacement of ^{3}H-Flu by CL-218,872. The displacement of ^{3}H-Flu binding by CL-218,872 was studied in our mice at different ages to examine the developmental profile of the Type I and Type II receptors in the cortex and cerebellum. When we examined cortical tissue, the IC_{50} value for CL-218,872 decreased in a biphasic manner from birth to adulthood (Fig. 4). During the first two weeks after birth, the IC_{50} value was reduced to approximately one third of the value noted at birth ($p<0.001$). From this point in time, the decrease in IC_{50} values were more

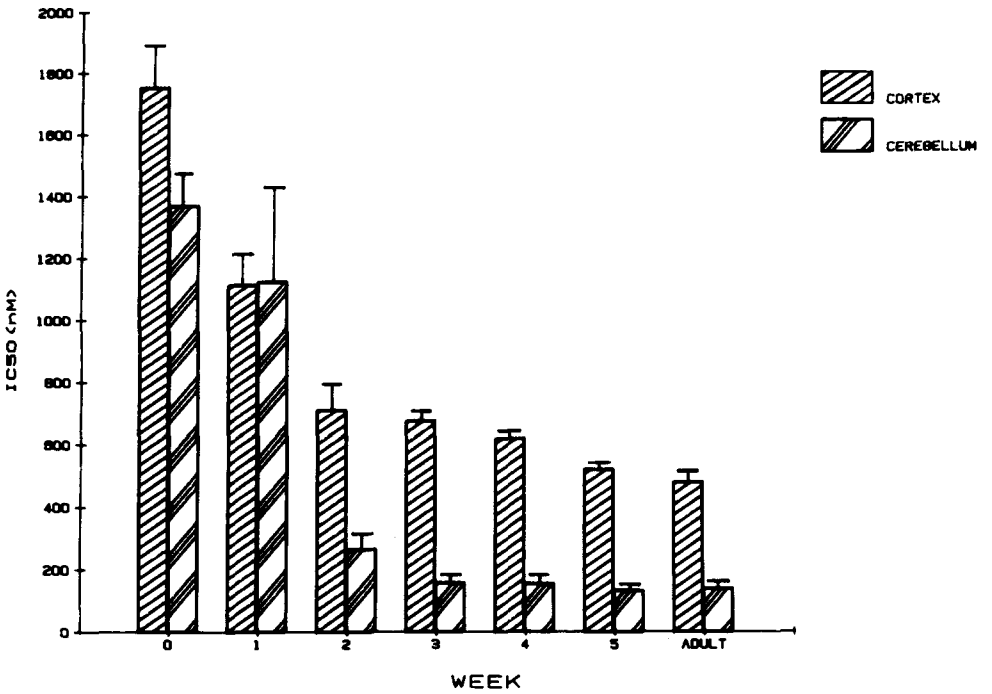

FIG. 4. IC_{50} values of CL-218,872 displacement of ^{3}H-Flu binding in the cortex and cerebellum. Studies examining the displacement of ^{3}H-Flu by CL-218,872 were performed by incubating membranes with 1 nM ^{3}H-Flu and varying concentrations of CL-218,872 (20–20,000 nM). The IC_{50} value of CL-218,872 was determined postnatally at weekly intervals in tissue from the cortex and cerebellum. IC_{50} values were derived by Hill analysis and the values represent the mean±SEM from four to eight experiments with each experiment containing tissue from 3–15 animals. Data was analyzed using ANOVA and the Student *t*-test (see text for results).

gradual, although significant (week three value compared to adult value, $p<0.005$) (Fig. 4). The cerebellar IC_{50} values for CL-218,872 exhibited a similar sharp decline during the first two weeks after birth ($p<0.001$). However, no statistically significant differences were noted between the IC_{50} values obtained at three, four and five weeks of age and the adult values in the cerebellum (Fig. 4).

Hill coefficients were calculated from CL-218,872 displacement curves and the developmental pattern for the Hill numbers was found to be significantly different between the cortex and cerebellum (Table 2). The Hill number in the cortex at birth equalled 1.0 and decreased ($p<0.001$) rapidly to reach the range of adult values by the time the animals were two weeks old. The Hill coefficient in the cerebellum at birth was 0.81 and did not change significantly with age.

The data obtained by use of CL-218,872 was subjected to a more rigorous kinetic analysis [16] which enables one to statistically evaluate the model (one- or two-site, etc.) which best fits the data and to determine the binding parameters of multiple binding sites.

LIGAND analysis of ^{3}H-Flu displacement by CL-218,872 from cerebellar membranes indicated that the data conformed to a one-site model at most of the test periods (Table 3). Data derived from cerebellar tissue of seven- and 21-day old mice did, however, conform to a two-site model ($p<0.05$) on a number of occasions (Table 3). The two-site model binding constants from the LIGAND program indicated that, at seven days of age, Type II receptors predominated in the cerebellum, while in 21-day old animals, the Type I BDZ receptor was the predominant entity in the cerebellum. Displacement data, which indicated that a one-site model was appropriate for characterizing BDZ receptors in the cerebellum (Table 3), was used to calculate overall K_D values for CL-218,872 displacement of ^{3}H-Flu binding. The K_D for CL-218,872 displacement of ^{3}H-Flu binding decreased from

TABLE 2

HILL COEFFICIENTS OF CL-218,872 DISPLACEMENT OF ^{3}H-FLUNITRAZEPAM

Week	Cortex	Cerebellum
0	1.00 ± 0.03 (6)	0.81 ± 0.04 (5)
1	0.91 ± 0.02 (7)	0.76 ± 0.07 (5)
2	0.75 ± 0.02 (8)	0.75 ± 0.04 (4)
3	0.78 ± 0.02 (6)	0.78 ± 0.02 (5)
4	0.78 ± 0.01 (4)	0.85 ± 0.02 (6)
5	0.76 ± 0.01 (4)	0.85 ± 0.04 (6)
Adult	0.71 ± 0.03 (6)	0.76 ± 0.03 (6)

Displacement of 1 nM ^{3}H-Flu by CL-218,872 (20–20,000 nM) was examined in cortical and cerebellar membranes postnatally at weekly intervals and in adult animals. The values represent the mean ± SEM of the Hill coefficients calculated from the displacement data which was derived from the experiments described in Fig. 4 and the text.

TABLE 3

POSTNATAL DEVELOPMENT OF TYPE I AND TYPE II BDZ RECEPTORS CHARACTERIZED BY DISPLACEMENT OF ^{3}H-FLU BINDING BY CL-218,872

		One-Site Model		Two-Site Model			
Age†	(n)	K_D‡	B_m§	K_D(I)‡	K_D(II)‡	B_m(I)§	B_m(II)§
Cortex							
0*	(6)	1,021 ± 107	11.7 ± 3.0	—	—	—	—
7*	(8)	795 ± 101	14.4 ± 0.8	—	—	—	—
14	(6)	—	—	42 ± 9	908 ± 59	11.9 ± 1.2	22.5 ± 0.8
21	(7)	—	—	55 ± 11	1,055 ± 97	24.6 ± 2.6	28.5 ± 3.7
28	(4)	—	—	101 ± 34	947 ± 81	19.8 ± 2.9	18.1 ± 2.3
35	(5)	—	—	88 ± 23	1,523 ± 357	21.8 ± 3.6	25.3 ± 3.2
Adult	(9)	—	—	77 ± 10	1,477 ± 173	30.5 ± 2.1	22.1 ± 1.9
Cerebellum							
0*	(6)	838 ± 90	14.4 ± 0.8	—	—	—	—
7	(5)	780 ± 82	7.4 ± 1.1	52 ± 10	2,042 ± 669	2.5 ± 0.3	4.4 ± 1.0
14*	(4)	430 ± 138	17.8 ± 2.1	—	—	—	—
21	(6)	137 ± 13	26.1 ± 1.8	64 ± 7	2,042 ± 489	26.0 ± 2.2	4.4 ± 0.9
28*	(6)	299 ± 44	29.5 ± 2.6	—	—	—	—
35*	(6)	311 ± 9	44.6 ± 6.2	—	—	—	—
Adult*	(6)	314 ± 36	31.8 ± 2.7	—	—	—	—

Data from studies of displacement of ^{3}H-Flu from specific membrane binding sites by CL-218,872 was analyzed by use of the LIGAND program and the reported values represent the mean ± SEM. The computer program was used to analyze whether the data best fit a one- or two-site model for binding of ^{3}H-Flu and CL-218,872 and was used to calculate the reported binding constants.

*Indicates the times during development when all data best fit a single-site model. At other ages a two-site model represented all the cortical BDZ receptor data significantly ($p<0.05$) better than a one-site model. With cerebellar tissue the data fit the two-site model in three of the five experiments performed with seven day old mice and in four of the six experiments performed with 21-day old mice. Thus, binding constants derived from both types of models are presented.

(n)=Total number of experiments at each point of development.

†Days.

‡nM.

§fmole/mg tissue.

birth to reach its lowest value at three weeks of age ($p<0.001$). Thereafter, the K_D values increased and attained adult values by four weeks of age (week three value compared to week four value, $p<0.025$).

LIGAND analysis of ^{3}H-Flu displacement by CL-218,872 in the cortex showed that the data best fit a one-site model at birth and at one week of age, but by the second week after birth, the data consistently fit a two-site model ($p<0.05$). At two weeks of age, the K_D values for CL-218,872 at the two sites were significantly different ($p<0.001$) from each other and these K_D values did not change in a further significant fashion as the animal matured (Table 3). The number of Type I and Type II cortical BDZ receptors expressed on the basis of fmole/mg tissue did, however, change with age (Table 3). At two weeks of age, the earliest time at which the two receptor types can be resolved, the Type I cortical BDZ receptor number was 11.9 fmole/mg tissue. The number of Type I receptors increased significantly ($p<0.01$) during the third week of development, stayed constant during the third through fifth week, and increased again ($p<0.05$) between Week 5 and adulthood. The number of Type II BDZ receptors in the cortex at the second week of age was equal to the adult level and although the mean values for the B_{max} of Type II BDZ receptors fluctuated somewhat with age the values did not change significantly during the time that the animal matured. If one expressed the B_{max} values for cortical Type I and Type II BDZ receptors in units of fmole/mg protein (data not shown), the *total number* of Type I and Type II receptors varied little over time between values obtained at two weeks of age (1,910 fmole/mg protein) and adult values of (1,856 fmole/mg protein). When B_{max} values were expressed as fmole/mg protein, the proportion of Type I to Type II receptors in the cortex of two-week old animals was 63% and the proportion was diminished to 45% at approximately four weeks of age and remained at this value into adulthood.

DISCUSSION

Studies of the developmental patterns of specific binding sites for various ligands can provide information not only on the ontogeny of particular receptor populations but may also provide evidence for characterizing receptor subtypes which interact with particular ligands. After the initial description of brain BDZ binding sites [4,15] considerable evidence for BDZ binding site heterogeneity has accumulated [9, 12, 20]. The triazolopyridazines (e.g., CL-218,872) have been used to support the contention that certain areas of brain in adult rodents contain BDZ receptors which have similar affinities for ^{3}H-Flu, but have significantly different affinities for CL-218,872 [9,11]. Based on such observations [11], the BDZ receptors were classified as Type I receptors which have a

high affinity for both ^{3}H-Flu and CL-218,872 and Type II receptors which have a high affinity for ^{3}H-Flu, but display a low affinity for the triazolopyridazines, including CL-218,872.

To provide further evidence in support of the presence of Type I and Type II BDZ receptors Chisholm *et al.* [6] examined the ability of CL-218,872 to displace ^{3}H-Flu binding in three regions of the rat brain at various times during postnatal development. Based on developmental changes in IC_{50} values for CL-218,872 and concomitant changes in Hill coefficients determined from their binding data, Chisholm *et al.* [6] concluded that Type I BDZ receptors displayed a developmental profile which differed from the developmental profile of Type II BDZ receptors. Type I BDZ receptors in the cortex and cerebellum of the rat seemed to develop primarily between the first and second week of postnatal life.

In examining the IC_{50} values for CL-218,872 in the cortex and cerebellum of the developing mouse we noted that in both brain areas the IC_{50} values were significantly diminished between birth and when the animals were two weeks of age (Fig. 4). On the other hand, the Hill coefficients, for displacement of ^{3}H-Flu binding by CL-218,872 calculated at different stages of development, differed between cortex and cerebellum and also differed from the pattern presented for the developing rat brain [6]. In our studies, the Hill coefficient determined with cortical tissue at birth was one, indicating a homogenous population of receptors for CL-218,872. The Hill coefficient determined with cerebellar tissue was however, significantly ($p<0.001$) lower than the value determined using cortical tissue. Low Hill coefficients can indicate negative cooperativity as well as receptor heterogeneity, but a number of experiments (detailed in [11]) have indicated that negative cooperativity can be ruled out as an explanation of low Hill coefficients in studies of displacement of ^{3}H-Flu by CL-218,872. One could, thus, hypothesize that cerebellar tissue of mice contains a heterogeneous array of BDZ receptors even at birth. During the maturation of the mice the cortical Hill coefficients declined with time to reach the adult values of 0.71 (Table 2). The major change in the cortical Hill coefficients was evidenced between the ages of one and two weeks. The cerebellar Hill coefficients changed little with age and adult values (0.76) were only slightly lower than the values obtained at birth. Similar studies [6] with rat cerebellar tissues determined the Hill coefficients to be close to one both at birth and when the animals reached adulthood.

Another means by which heterogeneous populations of receptors can be characterized is to subject the data on the displacement of ^{3}H-Flu by CL-218,872 to analysis by the weighted, least squares, curve fitting program, LIGAND [16]. Given significant differences in ligand affinity constants at the different receptor sites and the presence of a substantive proportion of various receptor types within the overall population of receptors, the LIGAND program can provide statistical information regarding the model (one-site, two-site, etc.) that best fits the data. The program also provides the kinetic constants for ligand binding at the various receptor populations. The figures in Table 3 indicated that binding data for CL-218,872 in the cortex fits a two-site model significantly better than a one-site model when the mice are two weeks of age and older. The ontogeny of the second site with high affinity for CL-218,872 is in accord with the data in Fig. 4 which illustrates a significant decrease in IC_{50} values for CL-218,872 displacement of ^{3}H-Flu binding in the cortex over the first two weeks of life, and the rather constant values obtained thereafter. The ontogeny of a second type of receptor in the cortex during the first and second postnatal week is also in accord with the calculated Hill coefficients (Table 2) which in the cortex, approximate the adult values when the animals reach two weeks of age. In examining the data in Table 3 one finds that the affinity of the binding sites for CL-218,872 in the cortex remains constant once both receptor sites become evident. The difference in affinities for CL-218,872 at the two sites is 10- to 15-fold. This difference corresponds to the difference in affinities for CL-218,872 at Type I and Type II BDZ receptors described in rat brain [26].

When one examines the data generated by the LIGAND program from studies performed with cerebellar tissue the presence of multiple binding sites for CL-218,872 is less clear. The changes in the IC_{50} values for CL-218,872 displacement of ^{3}H-Flu binding during development (Fig. 4) and the Hill coefficients which differ from unity (Table 2) would lead one to suspect the presence of heterogeneity in cerebellar binding sites for CL-218,872. On the other hand, a major portion of the CL-218,872 binding data during cerebellar development better fit a one-site model for CL-218,872 binding when such data was processed by the LIGAND program. If one accepts the kinetic constants derived by the LIGAND program for the single-site model, the affinity for CL-218,872 at the cerebellar BDZ receptor increases with the age of the animal (Table 3). The affinity of this receptor for flunitrazepam would, on the other hand, be diminishing during development (Fig. 3). One would be led by this data to consider progressive modification of the BDZ binding sites in the cerebellum which would impart to the receptor its particular ligand binding characteristics. These characteristics would be distinct from the K_D's calculated for either Type I or Type II BDZ receptors in the cortex. Such a conclusion would not be supported by experiments which illustrate ^{3}H-Flu binding proteins of similar characteristics being derived from various brain areas [21,22]. An alternate explanation can, however, better reconcile the data without the need of generating an additional cerebellar type BDZ receptor. The explanation would be based on the probability that the proportion of either Type I or Type II receptors in the cerebellum of the HS mice during the various stages of development is too low to allow the receptor types to be distinguished by the LIGAND analysis. In examining the ability of iterative programs to distinguish receptor subtypes, Minneman *et al.* [14] demonstrated that two receptor subtypes are best distinguished when the ratio of the subtypes in a particular tissue is approximately one and/or the receptor-selective ligand differs substantially in its affinity for the two receptor subtypes (e.g., 20- to 100-fold). Given a difference in affinities for CL-218,872 of 10- to 18-fold at Type I and Type II BDZ receptors, the presence of a low proportion of one of the receptor types in the cerebellum would result in the generation of a statistically better fit of the data to a one-site model under our experimental conditions. The single binding constant generated by applying this model would, however, reflect the contribution of both ligand binding sites [16].

At two points during the development of the cerebellum, data for displacement of ^{3}H-Flu binding by CL-218,872 was found to fit a two-site model in a statistically significant fashion (F values < 3.8, $df=12$). This occurred in three of the five experiments performed with tissue of seven day old animals and in four of the six experiments performed with tissue from 21-day old animals. The results of these experiments indicate that Type II BDZ receptors exceed, in number, the Type I

receptors in tissue of seven day old animals and the situation is reversed by the time the animals have reached three weeks of age. Examination of data (Table 3) regarding the two binding sites for CL-218,872 in both cortex and cerebellum would lead to the proposal that Type II receptors are present at birth and the concentrations of these receptors (fmole/mg tissue) changes relatively little during development. Type I receptors, on the other hand, proliferate most rapidly from birth through the second to third week of life. Although this proliferation of Type I receptors takes place in both cerebellum and cortex, the proportion of Type I to Type II receptors in the cerebellum of three week old mice, and, most probably, adult mice is substantially greater than the proportion of Type I to Type II receptors in cortical tissues of three week old or adult animals. The predominance of Type I receptors in the cerebellum of adult mice would be in accord with prior investigations on the distribution of Type I and Type II BDZ receptors in rodent cortex and cerebellum [10,11].

The data in Table 3 regarding the tissue concentrations of Type I and Type II receptors corresponds well with the data regarding the ontogeny of ^{3}H-Flu binding sites in cortex and cerebellum (Table 1). The sum of Type I and Type II BDZ receptors closely parallels the total concentration of ^{3}H-Flu binding sites (fmole/mg tissue) during all stages of development. If one accepts the proposal that ^{3}H-Flu labels both Type I and Type II BDZ receptors one could propose from our data that the Type I receptors have a lower affinity for ^{3}H-Flu than do the Type II receptors and thus the overall increase in K_D for ^{3}H-Flu binding in the cerebellum (Fig. 3) would be a reflection of the proportion of the Type I receptors in this tissue at various times after birth. Recent data on affinities of rat brain Type I and Type II BDZ receptors for ^{3}H-Flu has demonstrated that Type I receptors have approximately four-fold lower affinity for ^{3}H-Flu as compared to Type II receptors [26]. Our data on the K_D for ^{3}H-Flu binding in cortical tissue during development also partially supports the above contentions. One would expect, however, that the K_D for ^{3}H-Flu binding would be at its lowest value at birth since this would be the time at which the Type II BDZ receptor is the predominant form of BDZ receptors in the cortex (Tables 2 and 3). Thus, other factors may contribute to the K_D values for binding of ^{3}H-Flu to cortical tissues at birth.

Past studies [1, 2, 5, 10, 17, 19] of the postnatal ontogeny of ^{3}H-Flu binding sites in various areas of brain have primarily used rats as experimental animals and data on the concentration of ^{3}H-Flu binding sites has been presented both as fmole/mg protein and as fmole/wet weight of tissue. With either mode of data presentation the total concentration of ^{3}H-Flu binding sites increases with age. The B_{max} values at birth were approximately 24 to 50% of adult B_{max} values when either cortex [1, 5, 10] or cerebellum [19] was examined. Our data on the ontogenic development of ^{3}H-Flu binding sites presents a somewhat different picture. If data was expressed on the basis of fmole/mg tissue then the concentration of cortical ^{3}H-Flu binding sites at birth was approximately 15% of the adult values. Cerebellar concentrations of ^{3}H-Flu receptors (fmole/mg tissue) were approximately 46% of the adult values. On the other hand, when data was expressed as fmole ^{3}H-Flu bound/mg protein (Table 1) the concentration of ^{3}H-Flu binding sites in the cortex, at birth, was equivalent to adult values and the concentration of ^{3}H-Flu binding sites in the cerebellum at birth (fmole/mg protein) actually exceeded the adult values. To reconcile the data on the number of ^{3}H-Flu binding sites/mg protein obtained in our studies with the data reported for rat brain, one would have to contend that rat brain membranes contain, at birth, approximately the adult compliment of protein and that receptors added after birth (e.g., BDZ receptors) produce little change in the membrane protein concentration. In the mouse brain, however, membrane protein concentration changes significantly with age (Fig. 2) and this increase in membrane protein concentration occurs in parallel with the increase in BDZ binding sites.

In summary, our data details the ontogeny of Type I and Type II BDZ receptors in mouse brain and presents the kinetic characteristics of these receptors during developmental stages from birth to adulthood. Our studies detail the proportion of Type I and Type II BDZ receptors in cortex and cerebellum at various stages of development and indicate that species dependent differences may exist in these proportions (e.g., see [6] for Hill coefficients determined with rat brain tissue). Our work also indicates the importance of the method used for expressing data on receptor development in mouse brain. Since the one other paper concerning the ontogeny of ^{3}H-Flu binding in mouse brain presented data based only on fmole ^{3}H-Flu bound/mg tissue, our data extends the base for comparison of ^{3}H-Flu binding between species and also allows for comparisons of the characteristics of Type I and Type II BDZ receptors during brain development. The presented information on the developmental profiles of the Type I and Type II BDZ receptors should aid in designing studies to elucidate both the pharmacological and physiological significance of the BDZ receptor subtypes. Such information should also aid in the identification of cell types containing the various BDZ receptors, and in attempts to separate and characterize the receptor subtypes by biochemical techniques.

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